STUDIES ON TYPHUS FEVER

II. Studies on the Etiology of Mexican Typhus Fever

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Plates 32 to 34

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In experimental typhus fever of guinea pigs the differences between the European strain and the Mexican strain are sufficiently definite to permit differentiation. The chief points which make such recognition possible were emphasized by Mooser (1) and have been confirmed in every particular by ourselves. They consist especially in the regularity with which testicular swelling occurs, after intraperitoneal inoculation, in the Mexican variety, the constant presence of the *Rickettsia*-like bodies described by Mooser in the tunica and the relatively earlier temperature rise which, in the Mexican virus animals, may begin on the fourth, fifth or sixth day, whereas in the European type it is usually deferred until the ninth day or later. In the European type, also, the temperature is apt to reach a higher point and, as Mooser correctly points out, the nodular lesions in the brain described by Otto and Dietrich (2), Spielmeyer (3), Wolbach (4) and others are more frequent. It should be noted, however, that if the Mexican virus is subcutaneously injected instead of intraperitoneally, scrotal swelling with *Rickettsiae* does not appear, the temperature is apt to begin later and the characteristic brain lesions are present. Subcutaneously inoculated Mexican virus, then, produces a disease closely simulating the European variety. Moreover, even after intraperitoneal inoculation, certain strains of the Mexican disease (like the "J" strain described by one of us in a previous communication) will occasionally produce disease without tunica lesions and with temperature curves and other features closely analogous to the European type of disease.

Common to the two diseases is demonstrable louse transmission (5), cross immunization and, under proper conditions, the brain lesion. In the European disease, also, we have been able to confirm Pinkerton's (6) observation that *Rickettsia* bodies similar to those described by Mooser can be found if the examination is made at the proper time. Failure in finding them, we believe, has been due largely to the fact that search has been deferred until the temperature rise has begun, a period at which the organisms have disappeared from the tunica. Why it is so much more difficult to find *Rickettsiae* in the tunicas of the European variety, we have no idea. Summarizing all these facts, however, it seems reasonable to assume that we are dealing with two closely related varieties of a single disease group into which, also, Rocky Mountain spotted fever may be placed, Mexican typhus fever occupying a position, as far as guinea pig inoculation is concerned, midway between the European typhus and Rocky Mountain spotted fever.

This of course does not justify one in applying all facts discovered about Mexican typhus fever to the European variety, but it does render it extremely likely that there should be etiological analogy and that immunological observations made on one disease can probably be applied by proper experimental methods to the other.

The work recorded in the present paper has been done almost entirely with the Mexican variety and deals primarily with the etiological problem. The causative significance of *Rickettsiae* has been advocated by many observers in the past, has been rendered likely by studies with lice and has been considerably strengthened by the discovery of these organisms in the tunica of Mexican typhus animals by Mooser. It is further supported by the experimental louse transmissions recently reported by this writer with Dummer (5) in which lice were infected by feeding on monkeys inoculated with Mexican typhus and developed organisms indistinguishable from those seen in the guinea pig. Although the methylene blue-saffranin method has shown that these bodies stain as easily as do bacteria, there is no doubt of their identity with the Giemsa-staining *Rickettsiae*, because of morphological similarities, intracellular position and resistance to cultivation. It may well be that cultivation may be accomplished in the future, and if so, the *Rickettsiae* may be recognized as a specialized variety of true

bacteria which have been modified by normal habitat in insects. Until this is accomplished, however, the Mooser bodies must be regarded as true *Rickettsiae* and this class of organisms segregated for purposes of convenience.

In spite of the probability with which the etiological importance of these organisms has been indicated by many investigations of the past, the crucial etiological demonstration is still lacking and the literature is confused by suggestions of filterable virus, mutations of proteus organisms and the suspicion that possibly the *Rickettsiae* or Mooser bodies represent merely a concomitant infection carried along with the typhus virus. Objections to the acceptance of *Rickettsiae* etiology have been based largely upon the uniform failure of attempts to find the *Rickettsiae* in virulent blood, organs and brain lesions; and the usually small numbers of visible *Rickettsiae* in tunica lesions, together with their intracellular position, has made it impossible to carry out inoculation experiments with material that did not contain, in addition to *Rickettsiae*, blood plasma and cells. The results obtained in our own laboratory in the last few years, which we think have strengthened the probable etiological significance of the Mooser bodies, have consisted in negative filtrations, the relatively greater degree of virulence of material containing large numbers of these organisms, the encouraging results of active immunization with formalinized tunica and the typical infectiousness of lice injected by the Weigl method with spleen material in which no *Rickettsiae* could be found by direct examination. Also, one of us has recently shown that the tunica lesion could disappear for several inoculation generations only to come back from time to time in individual guinea pigs, in every case with the presence of *Rickettsiae*.

It is not impossible that the peculiar pathology of the disease in guinea pigs could be explained by the fact that, on intraperitoneal inoculation, the *Rickettsiae* accumulate in the tunica, perhaps because of favorable temperature conditions or nutritive and cellular selective advantages and, from there, penetrate the body generally, disappearing from the tunica as the disease becomes generalized.

The present paper is a record of experiments in which we have endeavored to increase the susceptibility of animals to Mexican typhus fever in order to investigate with greater accuracy the dis-

tribution of the *Rickettsiae* or Mooser bodies throughout the organs, and in order to obtain these microorganisms in sufficient numbers to enable us to carry out a crucial etiological experiment.

Our first attempts were based on the reasoning that the testis was of a lower temperature than the body as a whole. The supposition was that perhaps the organisms were carried through the peritoneum into the tunica, and there found lower temperature conditions. The experiments carried out on this plan consisted in the use of two methods to lower the temperature of guinea pigs—one by keeping them, after inoculation, in a cold room having an average temperature of 5° to 8°C.; the other in lowering the general temperature by feeding considerable amounts of methyl alcohol by mouth. The latter method was by far the more successful, actually bringing the temperature of guinea pigs down to 97° to 99° during a period of prolonged intoxication, and appearing to give us a much more plentiful *Rickettsiae* yield in the tunica. Neither of these methods, however, was adequate for our purposes.

While studying tunica lesions and exudates by the methylene blue-saffranin method, we frequently saw actual phagocytosis of *Rickettsiae* by polyomorphonuclear neutrophiles. A drawing of such a condition is shown in Fig. 1. This, we believe, accounts for the occasional slight infectiousness of washed leucocytes recorded in a previous paper (7). It also suggested to us that possibly the destruction of *Rickettsiae* in the body was actively carried on by the mobile phagocytes, and that an inhibition of phagocytosis might give us better results. In consequence, we turned to the use of benzol, as recommended by Weiskolten, Schwartz and Steensland (8).

We carried out experiments with benzol both in guinea pigs and in rats, injecting subcutaneously mixtures of equal volumes of benzol and olive oil and after several such treatments—or at the same time with these treatments—inoculating intraperitoneally with tunica material containing considerable numbers of the organisms.

The benzol injections have given us remarkable results in four or five animals up to the present time, with less successful experiments in a number of others. We have still a great deal to learn about the amounts of benzol that can safely be injected, the intervals of injection, and particularly the most advantageous time after adminis-

tration of the benzol at which the infectious material should be injected into the prepared animals. These points are being subjected to detailed study at the present time. Meanwhile, the successful animals have given us results which we believe to be of the greatest importance in connection with the etiology of typhus fever.

An example of one of the best of these benzol rat experiments is the following:

Experiment—Benzol Rat IA.—This rat was subcutaneously injected on May 12 with 2 cc. of a mixture of equal parts of benzol and olive oil. 2 days later, on May 14, this animal received intraperitoneally an injection of tunica of Guinea pig 1, moderately rich in both intra- and extra-cellular *Rickettsiae*. On May 18, when the rat was to all appearances quite sick, it was chloroformed, carefully dissected and smears taken from the tunica, peritoneal exudate and peritoneal cells, spleen, liver, brain, bone marrow and blood. Examination of these smears showed enormous numbers of intra- and extra-cellular *Rickettsiae* in both tunica and peritoneum; *Rickettsiae* were seen both intra- and extra-cellularly in the spleen and in the liver; they were also present in certain elongated cells in the brain smears, possible from the pia, and were found in small numbers in the blood.

In another animal similarly treated, except that two injections of benzol were given, the organisms were similarly distributed, except that while tunica and peritoneum were less heavily infected, the liver contained great numbers grouped in clumps and there were organisms extra-cellularly in the blood and—in one field—crowded in a mononuclear cell which, being found in the blood, might well be a desquamated capillary endothelial cell.

In another rat in which several benzol injections had been given, a similar result was obtained and the rat died on the fifth day after inoculation. The death of this animal, judging from the distribution of *Rickettsiae*, might well be regarded as having been due to these organisms, in which case we may have succeeded in possibly converting typhus into a fatal infection for rats. It is impossible, however, to be sure of this, since the death of the animal might have been due to benzol or to a combination of the two causes.

Material for sections of all the organs was taken, and sections will be studied for the purpose of differentially localizing the organisms in the various cells. We would include these studies in the present report were it not for the fact that our differential stain has not yet been successfully adapted to tissue staining, and Giemsa is apt to give such poor contrasts that organisms as small as the *Rickettsiae* are likely to be visible only in certain cell groups which may not represent the total distribution

Figs. 2 to 7 are drawings and photographs made directly from slides of smears from two rats, showing the numbers and distribution of the *Rickettsiae*.

Experiment with the Inoculation of Washed Rickettsiae

The large numbers of extra-cellular *Rickettsiae* or Mooser bodies in the peritoneum of two of these rats gave us, we thought, an opportunity to carry out a crucial etiological experiment by washing the peritoneal exudate in Locke's solution and infecting guinea pigs with suspensions of *Rickettsiae* reasonably free from both blood plasma and cellular material.

Experiment I.—The peritoneum was washed with Locke's solution instilled through an incision in the peritoneal wall, about 15 cc. of Locke's solution being used. This material was centrifuged at a low speed for 5 minutes to remove most of the cells, the supernatant fluid again centrifuged for 5 minutes more to further remove cells, and then allowed to settle in the ice chest for a little less than two hours. Again at this time, the supernatant fluid was taken off and centrifuged at high speed for 1 hour. The supernatant fluid was then removed and the hardly visible sediment emulsified in several cubic centimeters of fresh Locke's solution. Two slides made from this suspension showed a not inconsiderable number of *Rickettsiae*, with only six cells that could be found by what we believe to be complete inspection of the two drops which had been allowed to dry on the slides. Two guinea pigs were injected subcutaneously with this material. Charts 1 and 2 made of these animals show only late and moderate rises in temperature, which are nevertheless suggestive of the typhus febrile movements often seen in subcutaneously inoculated Mexican typhus animals. These animals were intraperitoneally reinoculated with Mexican typhus blood on the 26th day, after the temperature had been normal for several days, together with a control. They remained normal, while the control came down typically, with a temperature of 104.2° and scrotal swelling on the sixth day, and on the seventh day a temperature of 106° and typical *Rickettsiae* in the tunica.

Experiment II.—Since we believed that in the first experiment attenuation of the *Rickettsiae* might have resulted from the length of time consumed in the washing, the exudate similarly taken from a second rat was centrifuged at low speed for 5 minutes, then the supernatant fluid from this centrifuged at high speed for 30 minutes. The sediment was again taken up in 15 cc. of Locke's solution, and again centrifuged at high speed for 30 minutes. This sediment was injected subcutaneously over the abdomen in Guinea pig 3. We specify the abdominal location of the subcutaneous injection because the subsequent testicular swelling in this animal might have been due to the escape of some of the injected fluid into the peritoneum. Our purpose in subcutaneously injecting was to produce, if possible, a European type of the disease, which would have been more convincing than a mere reproduction of the typical Mexican disease with the relatively pure *Rickettsiae* inoculum. As the chart of Guinea pig 3 shows, there was a typical rise of temperature, exceeding 104° on the twelfth day and almost reaching 105° on the

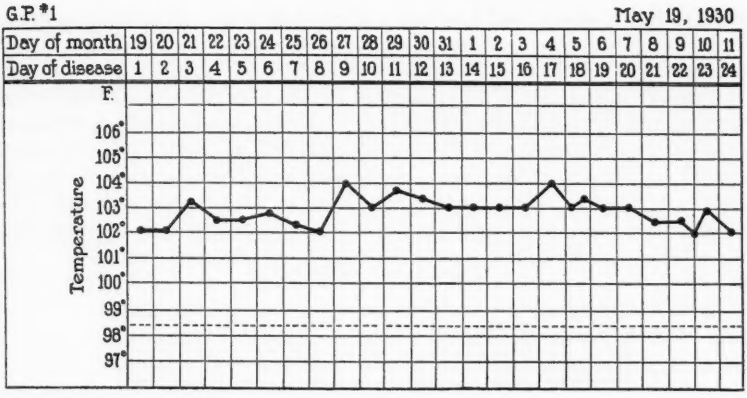

CHART 1

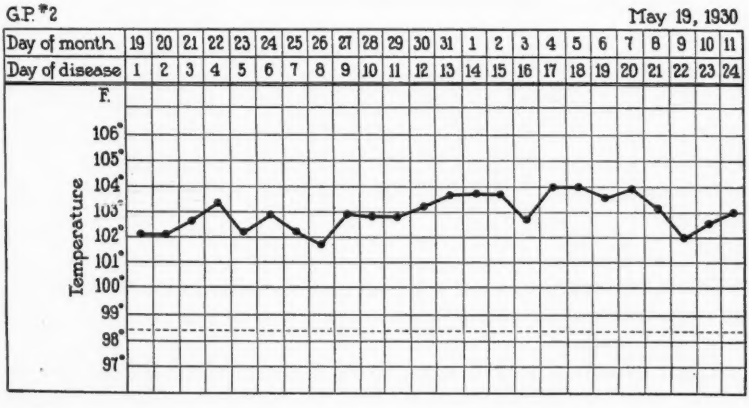

CHART 2

CHARTS 1 AND 2. The above charts are those of the two guinea pigs subcutaneously injected with thoroughly washed *Rickettsia* material in which microscopic examination showed many *Rickettsiae* and very few cells. The procedure took about 4 hours during which the material was about 1½ hours in the ice box. It is not unlikely that the *Rickettsiae* were attenuated by this procedure, which may account for the unconvincing fever curves. These animals, however, were re-inoculated with typhus blood on the 26th day and showed no reaction whatever, while the control came down on the 7th day with a temperature of 104.2° and 106° on the 8th day with marked swelling. The washed *Rickettsiae*, therefore, immunized against the typhus blood, which indicates identity of virus and *Rickettsiae*.

thirteenth, a condition, as far as fever curve is concerned, more analogous to the European than to the Mexican type. However, on the fourteenth day scrotal swelling appeared, the animal was killed, and *Rickettsiae* were found in the tunica.

Although it is more difficult in Mexican typhus fever to find brain lesions and although this animal was killed rather early in the disease for the demonstration of these pathological changes, the brain of this guinea pig was removed, sectioned and stained by Giemsa. There was a certain amount of proliferation of vascular endothelium and a number of lesions were found which we regarded as suggestive of the typhus ones, and two of which Dr. Wolbach—from a greater experience with the pathology of this disease—tells us he would accept as typhus lesions.

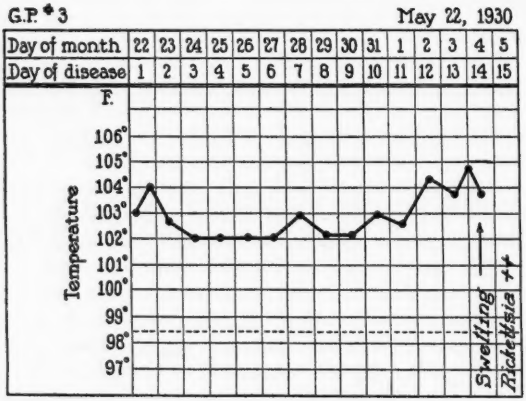

CHART 3. Temperature chart of Guinea pig 3, inoculated with washed *Rickettsiae* as described in Experiment II.

These experiments will have to be elaborated of course and repeated, but we publish them because we can see no obvious source of error, and we believe that we have reproduced in guinea pigs a disease which is clinically similar to Mexican typhus fever, including scrotal swelling and *Rickettsia* bodies, with material in which there might have been a very few endothelial cells from the peritoneum, but in which there was certainiy not enough blood plasma to cause infection and which consisted, as closely as it is possible to accomplish this at the present time, of washed *Rickettsiae* or Mooser bodies.

DISCUSSION

By the use of benzol preliminary to inoculation with tunica material of guinea pigs, we have succeeded in a number of rats in producing a

massive invasion of *Rickettsiae* or Mooser bodies, with large numbers in the tunica and peritoneum and considerable numbers in the spleen, liver, pia mater and in the blood itself. The manner of action of the benzol is as yet undetermined, but we believe that it depresses resistance by the diminution of phagocytosis by the polynuclear neutrophiles, which—from direct observation—we believe to be a powerful factor in the defence of the body against typhus fever. We have not yet succeeded in working out the quantitative and time factors by which these results can be achieved with regularity, but we have had perfect success in three instances and partial success in several others.

The benzol experiment, leading to a greater concentration of *Rickettsiae* in the blood, has made possible the bedbug feeding experiments described in our typhus Study III.

In obtaining large amounts of *Rickettsiae,* many of which were extracellular, in the peritoneum, we were enabled to carry out guinea pig inoculation experiments with washed *Rickettsiae,* free of serum and almost free of cells.

Two guinea pigs subcutaneously inoculated with such washed material, in which the process of washing had consumed about 4 hours, including over 90 minutes settling in the ice chest, presented atypical but—considering possible attenuation and the subcutaneous method of inoculation—probably true typhus reactions. This supposition is corroborated by the fact that these animals subsequently showed no reaction whatever to an intraperitoneal injection of Mexican typhus blood to which the control reacted typically in 7 days, with swelling and a temperature reaching 106°F. In a preceding paper we have presented evidence which suggests that guinea pigs can be actively immunized with formalinized tunica material. In such experiments, so far as our limited experience has gone, it was necessary to allow about a month to elapse between the last vaccine injection and the inoculation with living virus. In guinea pigs which have passed through a typhus infection immunity is established as soon as the temperature returns to normal. Since this was the case in these two animals, we believe that they had recovered from an attack of typhus conveyed by washed living *Rickettsiae.* In either case, the evidence is definite that the injected, washed *Rickettsiae* conferred typhus immunity.

The importance of this experiment lies in the fact that the subcutaneous injection of washed *Rickettsiae* from a Mexican typhus fever strain, free of plasma and almost entirely free of cells, produced a mild febrile reaction suggestive of, although not entirely characteristic of typhus fever. The mildness of the fever curve we attribute to the partial attentuation of the organisms in the course of washing. The absence of scrotal reaction was due to the subcutaneous manner of injection and renders the significance of the experiment rather greater than less. The fact that this inoculation with presumably pure *Rickettsiae* material protected these animals against an amount of virus which produced an unusually violent reaction in a control animal, with temperature, typical scrotal swelling and *Rickettsiae*, presents a train of evidence which in our opinion is incompatible with any interpretation other than that the *Rickettsiae* constitute the virus of Mexican typhus fever.

Another guinea pig, No. 3 (see Chart 2), after subcutaneous inoculation with washed *Rickettsiae*—a few of which probably filtered through to the peritoneum, since the injection was over the abdomen—developed a typical temperature curve with scrotal swelling and *Rickettsiae* in the tunica. This animal was killed on the thirteenth day, and though this was too soon to show well-developed brain lesions, a few lesions were found, which were histologically consistent with those described as characteristic of typhus by Otto and Dietrich, by Spielmeyer and by Wolbach.

CONCLUSIONS

We have adduced strong evidence in favor of identifying the virus of Mexican typhus fever with the *Rickettsiae*, or Mooser bodies, observed in the tunica lesions of Mexican typhus guinea pigs. Were it not for the possible presence of a few remaining cell fragments in the washings we would consider this evidence crucial proof.

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EXPLANATION OF PLATES

PLATE 32

FIG. 1. Drawing of phagocytosis of *Rickettsiae pr.* (Mooser bodies) in polynuclear leucocytes. Peritoneal exudate.

FIG. 2. Distribution of *Rickettsiae* in peritoneal scrapings.

PLATE 33

FIGS. 3 and 4. Photographs of peritoneal exudate in benzol, Rat IA, with cells crowded with *Rickettsiae* and extra-cellular organisms. (Kindly taken for us by Dr. Wolbach.)

PLATE 34

FIG. 5. *Rickettsiae* in liver, Rat IA.

FIG. 6. *Rickettsiae* in blood, Rat IA.

FIG. 7. *Rickettsiae* in elongated cells of pia from brain smears.

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(Zinsser and Castaneda: Typhus fever. II)

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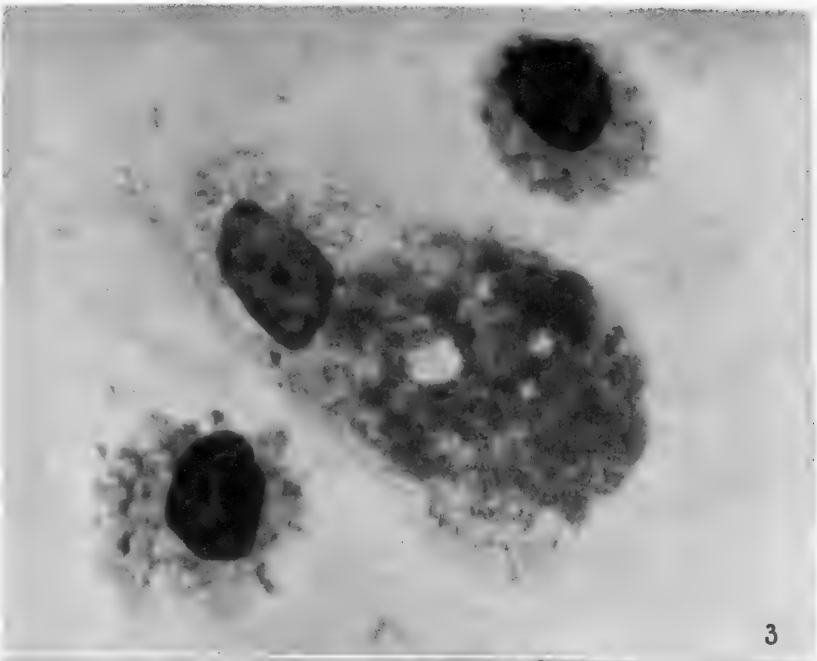

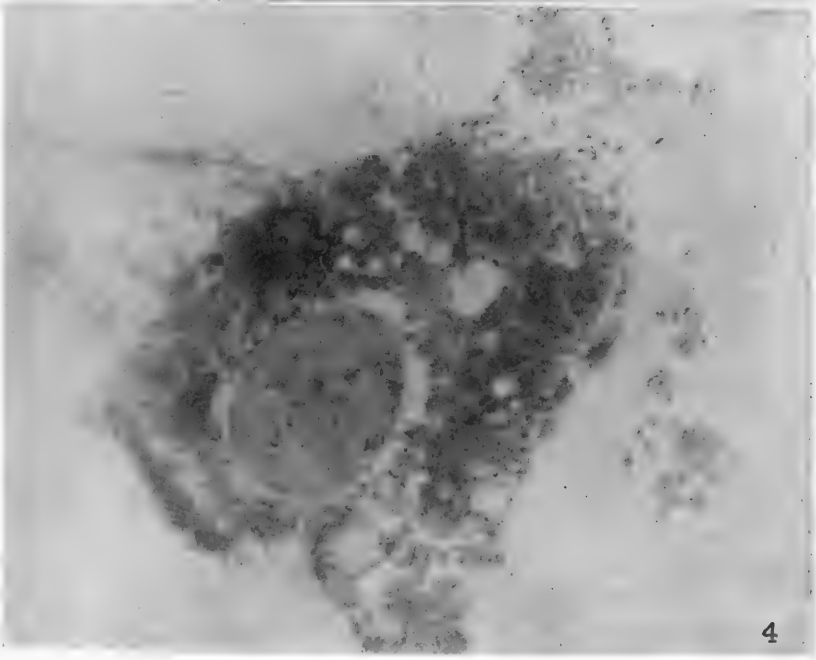

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STUDIES ON TYPHUS FEVER

III. Studies of Lice and Bedbugs (Cimex lectularius) with Mexican Typhus Fever Virus

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I

The Transmission of Mexican Typhus Fever through Lice Inoculated with Guinea Pig Organ Emulsions

One of the difficulties in the acceptance of Mooser bodies or *Rickettsia prowazeki* as the cause of typhus fever has been the fact that these organisms have not been found in the organs or blood of infected animals with which the disease can be transmitted. From titrations reported in a previous paper (1) it was quite obvious that inability to find the organisms in blood or tissues might well be due to their sparseness and the difficulty of recognizing them with certainty when isolated organisms are present in protein suspensions.

It would be strong evidence in favor of the presence of the *Rickettsiae* in the virulent organs not as incidental contaminations but as true etiological agents, if lice could be infected with such organ materials and from them guinea pigs inoculated, the disease produced and typical tunica *Rickettsia* lesions observed.

The actual injection of lice per rectum by the Weigl method is relatively easy, but it is not so easy to keep the lice alive after the injections, since guinea pig plasma and some of the guinea pig tissues seem to exert a toxic action to which lice succumb, although daily feedings on immune subjects are practiced. In consequence, out of seven experiments, only the seventh was successful, but there could be no possible error in this, the negative results being obviously attributable to technical difficulties.

The following brief description of all our experiments is given because some of the negative ones, we believe, have considerable value.

Experiments on Lice Transmission of Rickettsiae from Tissue

1. Feb. 8, 1930. Tunica of an infected guinea pig, containing many *Rickettsiae*, injected intrarectally into 20 lice. After 10 days' feeding, *Rickettsiae*, were found in the intestinal endothelium of a number of these insects. Guinea pig inoculation was not carried out with these lice.

2. Feb. 18. Brain suspension injected as above in 20 lice. Only 6 of these survived the 10 days' feeding. No *Rickettsiae* found, and no guinea pig injections done.

3. Feb. 27. 22 lice injected with virulent guinea pig plasma and human cells from defibrinated blood. Only 3 remained alive after 10 days, though regularly fed. No *Rickettsiae* found in these. The guinea pig control injected with the original plasma came down typically, with *Rickettsiae* in the tunica.

4. Mar. 19. 50 lice injected with human red cells which had been in contact with infectious guinea pig plasma and centrifuged. This was done on the basis of an experiment published in a preceding paper (1) in which it was shown that the typhus virus adhered tightly to the red cells after the exposure of normal cells to infectious plasma. The purpose of this was to eliminate the toxicity of the plasma. Only 2 of the lice lived 10 days. No *Rickettsiae* were found. Control guinea pig came down typically with *Rickettsiae* in the tunica.

5. Mar. 27. 42 lice injected with emulsion of suprarenal tissue from an infected guinea pig plus normal human red cells. After 10 days only 15 lice alive. No *Rickettsiae* found in smears and injected guinea pig was negative. The control guinea pig injected with the original material was positive, with *Rickettsiae* in the tunica.

6. Apr. 25. 25 lice injected with brain tissue. Of these only 16 survived 10 days. No *Rickettsiae* found in the lice examined. The guinea pig injected with them remained negative and the guinea pig injected with the original material remained negative as well.

7. May 8. 43 lice injected with a mixture of suprarenal and spleen tissue from an infectious guinea pig. 38 of these survived after 10 days' feeding. Since it is quite impossible to examine all the lice for *Rickettsiae* without ruining the material for injection, the ground suspension of a mixture of all the intestinal tracts was examined, but no *Rickettsiae* found. Two guinea pigs were injected with this suspension. One had a temperature of 105° with marked testicular swelling, on the 9th day and plentiful *Rickettsiae* in tunica smears (Chart 1). The other had fever on the 18th day. Temperature 105° and swelling on the 19th. No *Rickettsia* examination was made on this animal.

From these experiments it may be concluded that when the organs of a Mexican typhus guinea pig, during the infectious stage in which

morphologically no *Rickettsiae* can be found, are injected into lice by the Weigl method and these lice are then kept alive for 10 days by feeding on an immune human being, the *Rickettsiae* undoubtedly present in these organisms in small number survive and the intestines of these lice injected into a guinea pig will reproduce the original picture of the disease, including the typical *Rickettsia* lesions in the tunica vaginalis.

In the transmission of Mexican typhus from guinea pig to guinea pig with blood and organ emulsions by intraperitoneal injection, *Rick-*

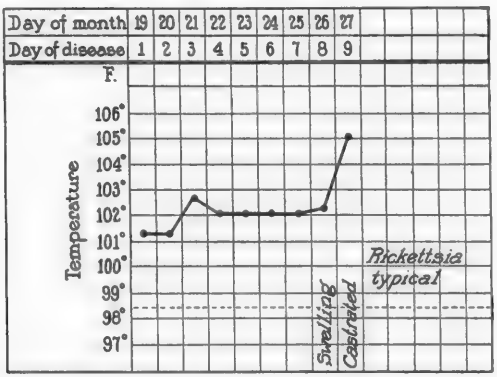


CHART 1. The above guinea pig was injected intraperitoneally on May 19 with an emulsion of lice that had been rectally injected with suprarenal and spleen tissue suspensions on May 8 and fed daily on an immune human subject in the interval.

ettsiae keep appearing in the tunica, and this alone would tend to indicate that these organisms were present in the organs without further demonstration. However, it may be—as it often has been argued—that possibly these *Rickettsiae* did not represent the actual virus but were incidental contaminations carried along with the virus.

Experiments like the successful one (No. 7) cited tend to indicate that the *Rickettsiae* present in the infectious organs can, like typhus virus, be carried for 10 days in infected lice and still be capable, after as many as eight to twenty normal feedings, of infecting guinea pigs in a typical manner. While this is not by itself conclusive proof of the identity of the Mooser bodies and the typhus virus, it adds another

link to the chain of favorable evidence, corroborating experiments like those of Mooser and Dummer (2) who found *Rickettsiae* in lice fed on Mexican typhus monkeys.

II

Experiments with Cimex lectularius

In the studies of Pinkerton in the Harvard Department of Pathology, as well as in our own studies and those of Maxcy, Dwyer and others, cross immunization and similarity of clinical and pathological findings in guinea pigs have identified the Mexican variety of typhus with the endemic typhus of the United States, which has been studied by Maxcy (3). Mooser has recently demonstrated the possibility of louse transmission with this disease, and undoubtedly such transmission can and does take place. On the other hand, the epidemiological studies of Maxcy indicate that, as he expresses it "There may be some mode other than direct transmission from man to man by means of the bite of a louse." In consequence, we determined to undertake the investigation of other insect possibilities in the transmission of Mexican typhus, and chose, as one of the most widely distributed and common, the *Cimex lectularius*, or bedbug.

Bedbugs collected from a non-typhus source—namely, in Boston—were kept in sealed Petri dishes with filter paper in the laboratory and first studied by dissection and staining of organ smears with the methylene blue-saffranin stain and with Giemsa. The normal *Rickettsiae* of the *Cimex* have been thoroughly described by Hertig and Wolbach (4), whose studies aided us considerably in a preliminary survey of the organs of these insects. Although morphological differentiations are not particularly reliable in determining differences between virulent and non-virulent *Rickettsiae*, yet as a guide to work of the kind we are describing, the illustrations and descriptions of the writers named, compared with our own observation of Mooser bodies or *Rickettsiae prowazeki* have given us some confidence in the belief that tentative differentiations, while not conclusive, are still useful. We mention this because later in the experiment to be described we believe that we saw the Mooser type of *Rickettsiae* in considerable numbers in infected bedbugs.

Early feeding experiments with bedbugs having been negative, it was thought desirable to determine the amount of blood which a bedbug can ingest, since we know that the blood of guinea pigs, even at the most favorable time for reinoculation, is not infectious in amounts smaller than 0.05 cc., and rarely infectious in amounts lower than 0.1 cc.

2 adult bedbugs which had been starved for a number of days were weighed. The weight of the bedbugs before feeding was 0.012 gm. These 2 insects were then allowed to feed on an infected typhus rat and not removed until they had had their fill. They were then again weighed in the same balanced watch glasses and were found to weigh 0.0305 gm.

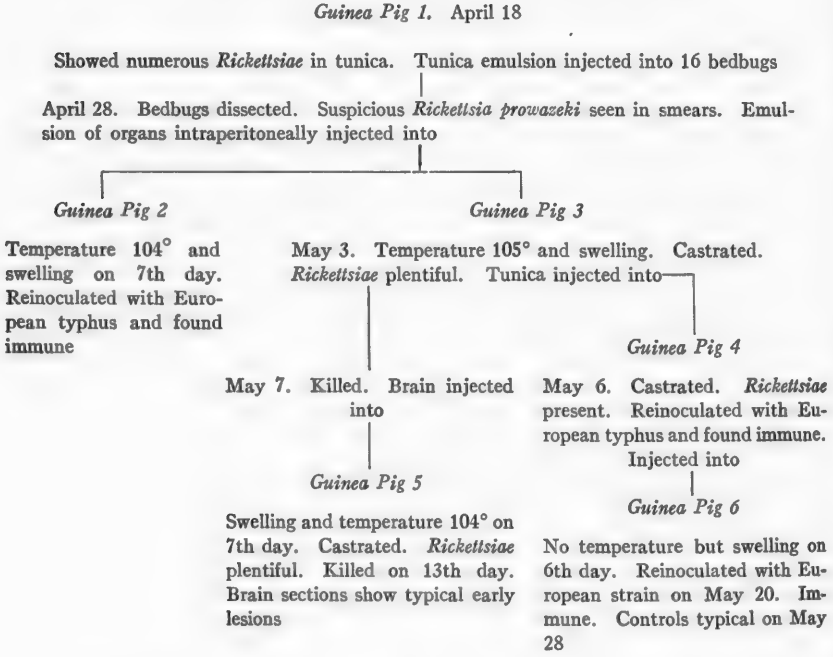
Subtracting the weight of the 2 bedbugs before feeding from their weight after feeding, and dividing this by 2, we obtain, as an approximate amount of blood taken up by each bedbug, 0.0093 gm., or slightly over 9 mg. of blood. Since it would then take approximately 10 bedbugs to take up a minimum dose for a guinea pig, we decided to carry out succeeding experiments by inoculating bedbugs with tunica material by a method analogous to the Weigl technique used in lice. This procedure is not very difficult, but the inoculated material passes into the coelom. In spite of this, however, the bedbugs survived.

Experiment on Injection of Bedbugs

Tunica material from Guinea pig 1, showing *Rickettsia prowazeki* or Mooser bodies on the fifth day after inoculation, was emulsified and injected with a very fine capillary pipette through the rectum into the coelom of 16 bedbugs. During the next 10 days they were fed three times on a normal guinea pig. This animal showed a slight fever for a day, but transfer from it did not produce typhus fever. 10 days after inoculation these bedbugs were killed, washed in 98 per cent alcohol and smears made from the gut and other organs. These smears, stained by the methylene blue-saffranin method, showed—in one *Cimex*—bodies resembling the typhus *Rickettsiae*. The organs of these bedbugs were emulsified and injected into Guinea pigs 2 and 3. Both these animals showed rises of temperature and typical swelling. Guinea pig 3 was castrated on the sixth day of the disease and the second day of swelling. Plentiful *Rickettsiae* were found in tunica smears, and inoculation was done with this material into Guinea pig 4. This animal showed a typical rise, with swelling and positive *Rickettsiae*, on the fourth day. Transfer was made from this to Guinea pig 6, which, in the third generation from the bedbug, showed scrotal swelling on the sixth day, but no temperature. This animal was kept for immunity test and inoculated on the sixteenth day with brain of the European typhus strain. It remained normal, although controls came down typically. Guinea pigs 2 and 4 were reinoculated with European typhus and found immune.

The original guinea pig, No. 3, which had been castrated on the sixth day after the disease, was killed on the eleventh day, when the temperature was still high, and carefully autopsied. There were no lesions of other disease in the organs. Cultures from the spleen were negative and material from the brain was injected into Guinea pig 5. This animal, on the seventh day after inoculation, had a temperature of 104° and swelling, and plentiful *Rickettsiae* were found in the tunica after castration on this day. On the thirteenth day this guinea pig was autopsied and typical brain lesions were found. These have been verified for us by Dr.

Wolbach, to whom we are grateful for his helpful interest in these experiments. The results of this experiment are tabulated in the accompanying diagram.


Guinea Pig 1. April 18
Showed numerous *Rickettsiae* in tunica. Tunica emulsion injected into 16 bedbugs
April 28. Bedbugs dissected. Suspicious *Rickettsia prowazeki* seen in smears. Emulsion of organs intraperitoneally injected into
Guinea Pig 2
Guinea Pig 3
Temperature 104° and swelling on 7th day. Reinoculated with European typhus and found immune
May 3. Temperature 105° and swelling. Castrated. *Rickettsiae* plentiful. Tunica injected into
Guinea Pig 4
May 7. Killed. Brain injected into
May 6. Castrated. *Rickettsiae* present. Reinoculated with European typhus and found immune. Injected into
Guinea Pig 5
Guinea Pig 6
Swelling and temperature 104° on 7th day. Castrated. *Rickettsiae* plentiful. Killed on 13th day. Brain sections show typical early lesions
No temperature but swelling on 6th day. Reinoculated with European strain on May 20. Immune. Controls typical on May 28

Another experiment, similar to the above, was carried out with 25 bedbugs injected on Apr. 30. While they were being fed on the same normal guinea pig, on four separate occasions during an interval of 10 days, the feces were rubbed into the skin of this animal, but without result. Inoculation after 10 days of the organs of these bedbugs into a guinea pig resulted in typical disease with swelling and *Rickettsiae*. The disease was carried on from this animal through three generations.

Inoculation of Bedbugs by Feeding

After we had had the success in producing considerable accumulations of *Rickettsiae* in organs and in the blood in benzolized rats, as described in another paper, we thought it worth while to resume feeding experiments.

On May 16, 25 bedbugs were fed on benzol Rat IA on the third day after infection. On May 17, 10 of these bedbugs were again fed on a similar rat, IIB,

72 hours after infection. On May 18, 17 of the bedbugs were again fed on Rat IIB. On May 20 and 24, the surviving bedbugs were fed on a normal guinea pig. On May 25, the 20 surviving bedbugs were washed in 95 per cent alcohol, the intestines emulsified and injected into a guinea pig. Smears of these organs showed intra-cellular *Rickettsiae* suggestively but not conclusively similar to the Mooser bodies. This guinea pig, as shown in Chart 2, developed a typical temperature with swelling on the fourteenth day of disease and typical intra- and extra-cellular *Rickettsiae* were found in tunica smears.

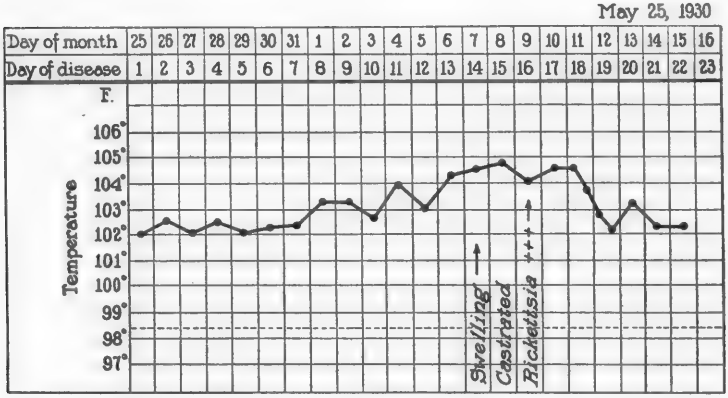


CHART 2. Chart of guinea pig inoculated with the organs of bedbugs which had fed upon benzolized typhus rats on the 9th, 7th and 6th days before the inoculation was done, and kept alive by feeding on a normal guinea pig in the interval. This guinea pig showed typical *Rickettsiae*, and was found immune to typhus on subsequent inoculation.

SUMMARY

Our experiments have shown that the Mooser bodies or *Rickettsiae* derived from guinea pigs with Mexican typhus fever can survive in bedbugs after intra-coelomic injection for 10 days, remaining capable of infection. We have also succeeded in similarly infecting bedbugs by allowing them to feed on benzolized rats in whose blood *Rickettsiae* had been shown to be present. Injection of the organs of such bedbugs 5 days after the last, 9 days after the first infectious feeding into guinea pigs produced typical Mexican typhus fever.

Some of the guinea pigs infected with such bedbug organs and pass-

ing through a typical typhus proved to be immune to subsequent inoculation with the European disease.

Attempts to infect normal guinea pigs by allowing infected bedbugs to feed on them or by rubbing the feces into the uninjured skin have, so far, been unsuccessful.

We have not, therefore, completed the cycle proving that bedbugs can transmit the disease, but we have shown that this is a possibility when dealing with man, obviously more susceptible to the disease than any of our experimental animals.

The ease with which the *Rickettsiae* seem to survive in the bedbugs suggests the desirability of investigating other common insects for a similar capacity of harboring the typhus *Rickettsiae*—experiments which we have not yet had the time to carry out.

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EXPERIMENTAL FIBROUS OSTEODYSTROPHY (OSTITIS FIBROSA) IN HYPERPARATHYROID DOGS

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Plates 35 and 36

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Ostitis fibrosa has been reported to occur spontaneously in animals other than man. Unsuccessful attempts have also been made to reproduce this condition by various experimental means. We have now produced the changes of true generalized ostitis fibrosa in guinea pigs and dogs (1–4). The experimental part of the present report describes in detail our results with dogs.

Spontaneous Occurrence of Ostitis Fibrosa in Animals

The bone changes of spontaneous ostitis fibrosa resemble in many ways, but frequently do not duplicate those observed in the fibrous osteodystrophies of man.

With regard to the appearance of osteoid tissue in the lesions observed in the spontaneous fibrous bone diseases of animals, Ingier (5) and Pick (6) stated that such lesions can be called ostitis fibrosa, even in the absence of osteoid tissue.

Peculiar to some animals with fibrosed bones is the common development of obstructions of the nasal and oral cavities, as these are encroached upon by thickening of their walls. As a result such animals may have sniffling respiration. However, fibrous bone lesions exist in animals not afflicted with sniffling respiration. Christeller (7), Rehn (8), Hintze (9), Busolt (10) and Ingier (5) described swine, goats, cattle and horses whose bones showed either true generalized ostitis fibrosa, or a local form of true ostitis fibrosa. In most instances only skulls were studied, but in a small number of cases complete examination of the skeleton was made. In these cases generalized ostitis fibrosa was found.

Koch (11) and Christeller (7) described spontaneous ostitis fibrosa in monkeys, Koch pointing out that this condition had been previously confused with rickets. White (12) detailed three cases of ostitis deformans in monkeys. Her cases were typical examples, both clinically and pathologically, of Paget's disease.

Dogs are also subject to spontaneous ostitis fibrosa. Christeller described a dog whose bones were thin and porotic. The long tubular bones showed curva-

ture and diminished longitudinal growth. In these bones the lesions were most prominent in the upper and lower portions of the diaphysis, where there was fibrous replacement of the marrow, transformation of the existing bone, new bone formation, bone resorption with numerous Howship's lacunae and osteoclasts. The haversian canals of the cortex were widened and invaded by fibrous tissue. The epiphyseal cartilage plates were present and relatively normal endochondral ossification was still in progress. The entire skeleton was involved, but the ribs showed relatively the greatest alterations.

Schmey (13) and Pick (14) described an atrophic deforming generalized bone disease in senile dogs with a predominance of osteoporosis, but with little fibrosis. Hager (15) described a case of ostitis fibrosa in a dog whose upper and lower jaws were tremendously enlarged. Weber's (16) dog, 8½ months of age, had spontaneous fibrous osteodystrophy with thickened porotic bones, cysts and giant-cell tumor formations. The jaw of his dog was also enlarged and cystic. The parathyroids were normal, and calcium metastases were not observed.

Attempts at Experimental Production of Ostitis Fibrosa

Many attempts have been made to reproduce experimentally all or some of the lesions of ostitis fibrosa—that is, cysts, hemorrhages, giant-cell tumors, marrow fibrosis, bone resorption and osteoid tissue.

Traumatization of the marrow has been extensively tried with the view of producing bone cysts or local ostitis fibrosa, but Lexer (17), Lotsch (18), and Nissen (19) have shown conclusively that injury, and the resultant hemorrhage into the marrow, do not lead to local cyst formation. Contrary to the opinion of Konjetzny (20) and Pommer (21), it seems logical to conclude that before an intramedullary hemorrhage results in local cyst formation there must be a generalized or an extensive local disease of the bone. The injection of fluids or the introduction of foreign bodies into the marrow cavity, injury of the bone or marrow by X-ray, by radium, by toxins developed in parabiotic animals, or by toxins of injected bacteria have led neither to true generalized ostitis fibrosa nor to true local ostitis fibrosa. The scarring, the marrow degeneration, or the cyst formation that may result from such treatment are only local reactive manifestations, having nothing to do with genuine ostitis fibrosa.

One-sided diets were among the first means employed in attempts at the experimental production of dystrophic bone diseases in dogs. Calcium deficiency was generally the underlying principle of the treatment. In most of the experiments the dogs received horse meat, fat or carbohydrates, and distilled water. The lesions produced in the various experiments were quite uniform. The older reports lacked detailed histological descriptions (22), and designated the changes produced as rickets or a ricket-like disease. Dibbelt (23) did not make a diagnosis.

It remained for Korssakoff (24) to point out that the condition produced in dogs on such a diet was osteoporosis and not rickets. Miva and Stoeltzner (25) studied

in great detail the changes produced by this diet and came to the same conclusion. They described marked osteoporosis with fibrosis of the marrow, but indicated that lacunar resorption was not severe enough to establish the lesion as ostitis fibrosa.

On a calcium-poor diet we, like some of the authors mentioned, have produced osteoporosis in dogs. These were fed lean meat but were protected against vitamin deficiency. On the other hand, in dogs which we maintained for a long period in a state of chronic hyperparathyroidism—both on adequate and calcium-poor diets—we produced generalized ostitis fibrosa. The clinical course, the evolution of the bone lesions, the gross and microscopical appearances of the bone and marrow were quite different in the two groups of animals, and indicated the specificity of the parathyroid extract treatment as the cause of experimental ostitis fibrosa.

The Relation of the Parathyroid Glands to Bone Dystrophies

Askanazy (26) was the first to suggest the etiological significance of a parathyroid adenoma found by him in a case of ostitis fibrosa. After his finding, many other instances of parathyroid enlargement were reported in association with ostitis fibrosa deformans, ostitis fibrosa cystica, osteomalacia, and rickets. Parathyroid enlargement has also been noted in rats suffering from experimental rickets.

Recently Barr and Bulger (27) summarized from the literature the cases with parathyroid tumors. They added 29 cases to those previously collected by Hoffheinz (28), making a total of 74 cases with pathologically enlarged parathyroid glands. These included both malignant and benign tumors, as well as enlargements due to simple hyperplasia. Authentic malignant growths of the parathyroids are extremely rare, and it is significant that none of them have been associated with evidence of functional derangement of the parathyroid glands as reflected in bone lesions. The majority of enlarged parathyroids reported are probably due to functional hyperplasia. The association of many diseases of bones with parathyroid hyperplasia or with benign tumor of the parathyroid glands is striking. Barr and Bulger state that 60 per cent of all cases of parathyroid enlargement had evidence of bone lesions. Clinically the most frequent as well as the most striking association is with the generalized form of ostitis fibrosa cystica—that is, the so-called v. Recklinghausen's disease.

The consensus of opinion until recently was that the parathyroid enlargement observed in association with these bone diseases was of a secondary nature, and appeared as a result of a compensatory hypertrophy due to the bone deficiency. In 1926 Mandl (29) brought clinical evidence to dispute this view. He removed a parathyroid tumor in a case of ostitis fibrosa cystica and reported rapid clinical

improvement of his patient, with decrease of urinary calcium excretion. He was the first to suggest, on the basis of his tests, that the skeletal changes in clinical cases were caused by a disturbed parathyroid function associated with the enlargement of the parathyroid gland.

Mandl's observations were soon confirmed and his conclusions widely accepted. A rapidly increasing series of reported cases supports the hypersecretion hypothesis. Gold (30), Barr, Bulger and Dixon (31), Barr and Bulger (27), Wilder (32), Boyd, Milgram and Stearns (33), Snapper (34), Richardson, Aub and Bauer (35), and Compère (36) reported cases showing a varying amount of improvement following extirpation of enlarged or even normal-sized parathyroids when hyperparathyroidism was diagnosed.

Hypercalcemia, and negative calcium balance when this has been demonstrated, may be attributed to the increased secretion of its active principle by the enlarged parathyroid gland. The case reported by Richardson, Aub and Bauer (35), by Bauer, Albright and Aub (37), by Hannon, Shorr, McClellan and DuBois (38), and by McClellan and Hannon (39) is important. In this case, which presented a clinical picture of hyperparathyroidism, no parathyroid tumor was found, and two normal appearing parathyroid glands were removed. The improvement in the condition of the patient was so marked that it indicated to these authors that hyperparathyroidism may result from hyperfunctioning of glands of normal size and appearance. The possibility exists that some undiscovered or abnormally active accessory parathyroid tissue may produce hyperparathyroidism in clinical cases where a tumor is not found after exploration.

A review of the symptoms in reported cases diagnosed as suffering from hyperparathyroidism, and of the clinical and chemical findings, reveals certain conditions common to all. Among these are: progressive muscular weakness, general lassitude and hypotonia; pain in and bowing of the lower extremities; resorption of the skeletal bones, frequently shown to be associated with hypercalcemia and with a negative mineral balance. Following operation the calcium balance, when this was studied, generally became positive. Fractures which occurred during the course of the disease healed very slowly (30, 31, 40) but soon after parathyroidectomy a fractured femur united firmly (34). Following removal of the parathyroid tumor, X-ray showed increased density of the bones in some of the cases (30, 31, 32, 34, 35). No positive evidence of healing of the bone cysts was included in the reported cases. Giant-cell tumors of the bones were noted in the cases of Wilder and of Barr, Bulger and Dixon, and these were found to be healed within a few months after removal of the parathyroid tumor.

Some of the reports cited above, available to us when our work began, indicated clearly that parathyroid adenomas or parathyroid hypersecretion were etiologically related to ostitis fibrosa cystica. We therefore felt that injections of parathyroid extract into experimental animals might produce bone lesions similar or analogous to

those found in clinical ostitis fibrosa, provided the animals could be maintained in a state of hyperparathyroidism without succumbing.

It is interesting to note that although a number of investigators injected parathormone into experimental animals for long periods, and although increased excretion of calcium at the expense of the bones is well established, no investigator has studied the bones of such animals histologically.

Experimental work with parathormone by Collip (41), Greenwald (42) and others had demonstrated that its injection led to hypercalcemia and to increased excretion of calcium and phosphorus, particularly in the urine; to lowered serum phosphorus and hypotonicity. Overdosage phenomena had also been described by Collip, Hueper (43) and Learner (44) in experimental animals (vomiting, diarrhea, hematuria, hemorrhagic gastro-enteritis, kidney impairment and hyperphosphatemia, and extensive metastatic calcification in the soft tissues, especially the kidneys, lungs and gastro-intestinal tract).

A careful study of the effects of daily injections of parathormone on rabbits, kittens and rats was made by Bauer, Aub and Albright (45). They were primarily interested in the question of whether the trabeculae serve as a reserve supply of calcium. After continued parathormone administration to rabbits for 91 days, during the major part of which they received 8 units daily, the trabeculae were reported as diminished in number, but no effect was noted on the cortex of the bone either in the gross examination or by X-ray. In growing kittens the administration of parathormone, on the average of 25.4 units daily for 56 days, did not result in any greater reduction of the trabeculae than observed in the course of normal bone growth. In the growing rat the administration of parathormone, 885 units over 110 days, resulted in diminution of the length of the bones and in an increase of the number of trabeculae. The serum calcium of the treated rabbits was higher than in the controls, while the serum calcium of the treated kittens was reported lower than in the controls, but there were no changes in the rats. They concluded, in spite of the fact that their own data do not seem to support their view, that bone trabeculae are easily depleted by prolonged administration of parathormone, and that these trabeculae serve as the source of readily available calcium.

Ingier (5), Christeller (7), Stenholm (46), Pick (6), Schmorl (47) and others emphasize the necessity of making histological studies of bone even in human cases, but especially in animal bone, before drawing conclusions regarding bone changes. Our own experience confirms

them. We have been frequently surprised at finding, upon histological examination, extensive resorption of cortical bone in our experimental animals, when the gross examination did not show anything obviously unusual. We believe that the results of Bauer, Aub and Albright, without the evidence of an histological examination, cannot be accepted as proving the absence of cortical resorption. Aub, Bauer and Albright based some of their conclusions on absence of hypercalcemia and on negative gross examinations. That these are not a proof of lack of parathormone effects is shown by our results in guinea pigs, which while receiving doses not large enough to produce hypercalcemia, still showed cortical bone resorption microscopically (1, 2, 48). In dogs as well (Nos. 3 and 4, see protocols), cortical resorption was produced on 2 units of parathormone daily for 20 days, although hypercalcemia was never observed.

Experimental Methods

We started with puppies 6 to 9 weeks of age, weighing 1 to 2 kg. The diet consisted of lean meat fed daily in amounts of about 15 per cent of body weight. Later, when active growth ceased, the amount fed was reduced to 10 per cent of body weight. These amounts corresponded actually to feeding *ad libitum*; the meat was frequently not consumed in full. Records of meat consumption were kept. The meat was supplemented daily with 1 cc. of cod-liver oil and at least 50 gm. of canned tomato. During various phases of the experiment known supplements of calcium were given in the form of calcium lactate by stomach tube, or calcium lactate and bone meal were added to the meat. The dogs were kept under carefully controlled hygienic conditions.

Serum calcium and phosphorus determinations were done at frequent intervals. The Clark-Collip modification of the Kramer-Tisdall method was employed in the calcium analyses, and the Benedict-Theis method in the phosphorus analyses. If the animal was receiving parathormone, the blood was generally examined about 18 hours after the last injection, the animals being fasted during that interval. The parathormone was injected subcutaneously in daily doses, which were increased as indicated by the course of the experiment.

The tissues and bones were fixed in neutral formalin or in Helly's fluid. The bones fixed in formalin were decalcified in Mueller's fluid plus 5 per cent glacial acetic acid. Those fixed in Helly's fluid were decalcified in 5 per cent nitric acid. When tissues were stained for calcium, they were fixed in 95 per cent alcohol and the v. Kossa method was used. Paraffin sections were always made. The details of these methods as employed in this laboratory have been described (49).

Experimental Results

After having gradually increased the daily dose of parathormone, we found that the dog could tolerate relatively large doses of the extract without showing hypercalcemia. We attribute the production of experimental ostitis fibrosa in dogs to the increased parathormone effects obtained by prolonged administration of relatively large doses of the extract. Otherwise the production in dogs of ostitis fibrosa would be difficult, because doses of parathyroid extract large enough to produce rapid resorption of the bone and injury to the marrow, lead in the dog to fatal hypercalcemia before there is much fibrous repair.

The parathormone was injected in a daily dose, at first usually of 2 units per kilogram, which was raised gradually in some dogs to as high as 5 units per kilogram towards the end of the experimental period of 5 to 6 months. Repeated small daily injections of parathormone at first usually raised the serum calcium to about 15 or 16 mg. per 100 cc. It was sometimes necessary to interrupt the parathormone treatment because of loss of appetite and other symptoms of overdosage. As treatment was continued, the serum calcium gradually fell to normal values or lower, and larger doses could be given with only a temporary elevation of serum calcium. This condition was produced most rapidly in one dog on a low calcium intake. In that dog (No. 8), serum calcium indeed dropped to as low as 7.8 mg. per 100 cc., and seemed to be associated with a tissue calcium lowered sufficiently to account for the tremors and tetany observed during the experiment (see protocol).

Immunity acquired as a result of previous parathormone treatment cannot be inferred from the normal serum calcium values in dogs treated for a long time with parathormone (Nos. 9 and 10), and from the hypocalcemia observed in one instance (No. 8). For when the calcium reserves were allowed to replenish after discontinuing parathormone for a short period, parathormone injections had their usual effect of raising the serum calcium (see protocols).

We produced, depending upon the dosage and the length of time under parathormone, all degrees of change from mild to severe bone resorption, and from slight fibrous replacement to degeneration of the marrow with hemorrhage, when the animals died from overdosage. Finally, we produced typical ostitis fibrosa cystica in several dogs. The most pronounced lesions were observed in three dogs that were

injected for 5 to 6 months. At the end of their experimental periods they were receiving 20 units daily.

One of the most marked clinical features in all these young growing dogs was the stunting of their growth, which was referable to a cessation of bone formation, and therefore of growth at the epiphyseal cartilage plates. After prolonged treatment the bones were deformed, and the forelegs showed lateral bowing. Such animals had pronounced hypotonicity of the muscles and developed plantigrade stance. In addition there was frequently torsion of the tubular bones on the long axis. The bone changes were generalized and lesions were found in the jaw bones, the skull, and all the long tubular bones studied. The ribs in the vicinity of the costochondral junctions offered the best material for study. In the long tubular bones, the fibrous changes were present only in the diaphysis and metaphysis. The epiphyses showed only a mild degree of simple resorption of the trabeculae and the marrow remained entirely free of fibrous changes. It is important to emphasize that none of our dogs had swellings at the epiphyseal cartilage plates, nor were there any indications of widened metaphyses or costochondral junctions.

In view of the frequent confusion in interpretation of the histological picture found in clinical or experimental bone dystrophies diagnosed as osteomalacia, osteoporosis and ostitis fibrosa, it is important to emphasize the criteria which must be satisfied by the lesion to which the term generalized ostitis fibrosa is applied. These are (*a*) resorption of the existing spongy and cortical bone, (*b*) invasion of the enlarged haversian spaces and of the marrow canal by fibrous tissue, and (*c*) the presence of Howship's lacunae, containing osteoclasts, on the walls of the haversian spaces, on the inner and outer surfaces of the compacta, and on the surfaces of the spongy trabeculae. New bone formation (osteoid tissue), as a substitute for the original lamellar bone, and cysts and hemorrhages in the marrow cavity, while frequently found, are in our opinion not essential for the diagnosis of ostitis fibrosa. Osteoid tissue appears as a result of the reparative process going on in bones subject to ostitis fibrosa, and may be absent, slight or extensive, depending on the functional need for reinforcement by osteoid repair.

Upon histological examination all the stated essential criteria of

ostitis fibrosa were found to be present in the dogs suffering from chronic hyperparathyroidism.

The fibrous bone changes were produced in dogs on a high or low calcium intake. In one dog (No. 7), receiving 650 mg. of calcium daily in the form of calcium lactate administered by stomach tube, considerable fibrous replacement of the bone was observed on a dose of 8 units of parathormone daily during the last 30 days of the parathormone treatment. The production of ostitis fibrosa was enhanced in the dogs by depleting their calcium stores more rapidly through the diminution of the calcium intake, which permitted giving larger doses of parathormone without danger of fatal hypercalcemia. When this condition is realized, an essential prerequisite exists for the production of ostitis fibrosa by parathyroid extract.

That the changes observed could not be attributed to calcium deficiency alone is evident from examination of bones of control dogs kept for months on a low calcium diet. These animals developed osteoporosis, which is histologically quite different from ostitis fibrosa cystica. Furthermore, by the use of parathormone, we have produced ostitis fibrosa in guinea pigs that were receiving a normal diet of adequate calcium content (1, 2).

PROTOCOLS

In the following protocols of 10 dogs on parathyroid treatment, only those data have been included which seemed necessary to a clear understanding of the means employed in the experimental production of ostitis fibrosa and of the results observed in the bones and tissues. The procedure had necessarily to be suited to each animal and was necessarily modified by developments. The central object was to preserve the life of the animal long enough to elicit the effects of experimental hyperparathyroidism on bone. A detailed presentation and discussion of the chemical data obtained will be published elsewhere, but sufficient data are given here to throw light on the clinical course of experimental hyperparathyroidism. The serum phosphorus data are given only when they indicate an exceptional condition. Some dogs died during the course of the experiments. With others, the experiments were terminated by administration of ether.

Dog 1.—Initial weight, 2.3 kg. Weight at the conclusion of the experiment, 11 days later, 2 kg. Calcium supplement, 600 mg. in the form of bone meal and calcium lactate. Four units of parathormone daily for 6 days and 8 units for 4 days. The dog ate all food until the 9th day, when complete anorexia appeared. The dog died on the morning of the 11th day. Serum calcium, 17.0 mg. per 100 cc. on the 10th day.

Autopsy showed loss of abdominal fat, involution of the thymus, and decrease of the external parathyroids to about one-half of the usual size. An hemorrhagic area was present in the left upper lobe of the lung; the kidneys were pale; the bones broke easily. Histological examination showed bone resorption, but the marrow changes formed the most striking feature. There was extensive necrosis of the marrow of all the bones examined. The marrow injury was most marked at the metaphyses and costochondral junctions. It was least pronounced or absent in the epiphyses. Fragmentation of the nuclei of the marrow cells was a prominent feature. The megakaryocytes were very badly degenerated and had entirely disappeared from some sections. Numerous mature neutrophilic and eosinophilic leucocytes appeared in the marrow. The erythrogenic cells were also degenerated. The connective tissue reticulum of the marrow was proliferated. There was diapedesis of red blood cells, particularly in the metaphysis, extensive dilatation of blood vessels, and considerable free marrow hemorrhage. Small collections of fibrin were observed in the hemorrhagic areas, and phagocytes also appeared in the marrow. The osteoclasts were large and numerous and for the most part not degenerated, indicating that they were reactive cells. In addition to the very extensive degeneration of the marrow, the osseous tissue of the ribs and tubular bones showed evidences of very rapid decalcification. The trabeculae and the walls of the haversian canals were nearly completely stripped of cells. The cartilage cores in the areas of endochondral ossification were either nearly bare or bare by the resorption of the previously deposited fiber-bone. New endochondral bone formation had ceased. This bone resorption was undoubtedly due mainly to vascular processes. Osteoclasts and Howship's lacunae were present, but mostly under the periosteum. Embedded and ground-disk cross-sections from the middle of the diaphyses of the long tubular bones showed enlargement of the haversian canals with Howship's lacunae containing osteoclasts. Examination of the soft tissues demonstrated extensive degeneration of the centers of the Malpighian corpuscles of the spleen, with congestion and leucocytic infiltration of the pulp; involution atrophy of the thymus; congestion of the lymph nodes; and congestion, leucocytic infiltration and pigmentation of the liver. The kidneys showed some calcium casts in the tubules. There was evidence of pneumonia, and the suprarenals were congested, with leucocytic infiltration. The tissues were not fixed or stained especially for calcium.

Summary.—This dog died of acute hyperparathyroidism. One of the most striking changes was in the bone marrow, which was ex-

tremely degenerated and hemorrhagic. The bones showed extensive decalcification. There had as yet been little marrow repair. The lymphoid tissue of the spleen was also degenerated.

Dog 2.—Initial weight, 1.65 kg. Weight at conclusion of experiment, 14 days later, 1.4 kg. No supplementary calcium was added to the diet. Four units of parathormone were given for 6 days, and 6 units for 3 days. On the 9th day the dog lost its appetite. On the 10th day it had bloody stools, and its serum calcium was 16.5 mg. per 100 cc. The dog died on the 14th day. Autopsy showed involution of the external parathyroids to about one-half the usual size and bilateral pneumonia and fibrinous pleurisy on the left side. Histological examination showed numerous degenerated kidney tubules containing calcium casts. Some glomeruli contained calcium deposits. The spleen was scarred, and showed evidence of active phagocytosis and leucocytic infiltration. The lungs contained patches of pneumonia and granules of deposited calcium. The bones were not studied. The parathyroids showed simple involution atrophy.

Summary.—This dog died of acute hyperparathyroidism complicated by bronchopneumonia. The soft tissues showed metastatic calcification.

Dog 3.—Initial weight, 1.95 kg. Weight at the termination of the experiment, 21 days later, 2.3 kg. Calcium supplement, 200 mg. daily, in the form of bone meal and calcium lactate. Injected 2 units of parathormone daily for 20 days. Serum calcium was 12.4, 11.7, and 12.8 mg. per 100 cc., respectively on the 11th, 18th and 21st days. The dog was killed to terminate the experiment. Autopsy showed a good state of nutrition. The organs and bones showed nothing unusual in the gross. The epiphyseal cartilage plates were thin. The thick cortex of the shafts of the long bones appeared compact in the gross. But when compared with a normal litter control, ground disks made from sections taken from the middle of the diaphysis of the long tubular bones showed definite microscopical evidence of resorption even of the apparently compact cortex. The Schwalbe and haversian canals were enlarged, but in stained sections no excessive connective tissue invasion of the canals was observed. In the stained sections of the long tubular bones no disturbance of endochondral ossification and no fibrous replacement of the marrow were found. The ribs, however, even with the small doses of parathormone received by this dog, showed degeneration of the blood-forming cells of the marrow, with destruction of the megakaryocytes which were being phagocytosed. There was fibrosis of the marrow, with the appearance of numerous leucocytes and eosinophiles. At the costochondral junctions rapid decalcification had taken place, and the more compact bone showed evidence of lacunar resorption with osteoclasts. The soft tissues showed nothing very unusual. Some kidney tubules contained calcium casts.

Summary.—Pronounced effects on the bone and marrow were observed even when parathormone was given for a short period in doses not sufficient to induce a hypercalcemia. The bone resorption was generalized and quite evident in the cortex of the diaphysis. The marrow changes were strikingly present in the ribs.

Dog 4.—Initial weight, 1.7 kg. Weight at the conclusion of the experiment, 21 days later, 1.95 kg. Calcium supplement, 600 mg. daily (bone meal and calcium lactate). Two units of parathormone were injected daily for 20 days. The serum calcium was 14.2, 12.4, 12.2, and 12.0 mg. per 100 cc., respectively, on the 11th, 12th, 18th and 21st days. The animal was killed to terminate the experiment when it was in excellent condition. Grossly neither the bones nor the soft tissues showed anything unusual. In ground disks of sections from the middle of the shafts of the long tubular bones resorption, as evidenced by enlarged vessel canals, was observed. Stained longitudinal sections of the long tubular bones showed some resorption of the compact and spongy bone, but connective tissue scarring was absent. The ribs, as in Dog 3, showed destruction of marrow cells, rapid decalcification at the costochondral junctions, osteoclasts and Howship's lacunae. There were calcium metastases in the kidneys. Otherwise the soft tissues showed no unusual features.

Summary.—In this dog, as in Dog 3, bone resorption and marrow fibrosis occurred after small doses of parathormone.

Dog 5.—Initial weight, 1.3 kg. Weight at conclusion of the experiment, 38 days later, 2.5 kg. Calcium supplement, 280 mg. daily for 5 days (in the form of bone meal), and 100 mg. (bone meal and calcium lactate) to the end of the experiment. The animal ate all its food until the 37th day, when it suddenly developed complete anorexia. The dog received 2 units of parathormone daily for the first 7 days, then 4 units daily for 17 days, 8 units daily for 4 days, 12 units daily for 6 days, and finally 16 units daily for 2 days. On the 37th day the parathormone was discontinued. At this time the dog was not obviously moribund. It was found dead on the 38th day. On the 12th day the serum calcium was 12.0 mg. 100 cc.; on the 28th, 11.6 mg ; and on the 34th day 11.0 mg. The blood obtained post-mortem showed a serum calcium of 13.6 mg. The dog probably died about 30 hours after the last injection. The urine obtained from the bladder at autopsy was acid (total titratable acidity equivalent to 660 cc. N/10 hydrochloric acid per liter). The autopsy showed that the dog was well nourished. There was considerable oedema of the subcutaneous tissues of the neck. The external parathyroids were reduced to from one-third to one-half their usual size. The lungs showed hemorrhagic and oedematous areas. The rest of the soft tissues presented nothing unusual in the gross. The bones were soft and brittle, and broke easily; the skull bones were easily cut with the scissors. Histological examination of the

bones showed rapid decalcification. The subepiphyseal cartilage plate trabeculae were reduced to cores of cartilage. New bone formation had ceased at the epiphyseal cartilage plates and costochondral junctions. The marrow was necrotic in places. Much of the cellular marrow had disappeared from the diaphysis. The marrow was fibrosed and contained leucocytes. The resorption of the bone was shown by the naked trabeculae and by the enlarged haversian canals. Numerous osteoclasts in Howship's lacunae were observed, but the hemorrhages and the vascular dilatations indicated that much of the resorption was vascular. While there was extensive resorption even in the shafts of the tubular bones, no osteoid tissue was observed in the marrow. The thymus was involuted. The lungs showed bronchitis and broncho-pneumonia and irregular collections of calcium were observed in the inflammatory exudate. The spleen was somewhat fibrotic and there was considerable necrosis of splenic cells. The parathyroids showed simple involutionary changes; the cells were to a great degree spindle-shaped.

Summary.—The dog undoubtedly died of hypercalcemic shock due to overdosage. It showed the acute effects of parathormone on the bone and marrow, superimposed upon the chronic effects of the previous treatment. A striking feature was the absence of osteoid tissue, which apparently does not appear until there is pronounced decalcification necessitating repair.

Dog 6.—Initial weight, 1.1 kg. The dog attained a weight of 2.1 kg. about 2 weeks before its death, which occurred 65 days after the beginning of the experiment. At autopsy it weighed 1.8 kg. The calcium supplement consisted of 280 mg. calcium in the form of bone meal for 5 days; of 100 mg. of calcium (bone meal and calcium lactate) for the next 36 days; it was increased to 1200 mg. for 5 days; no calcium was given for 2 days, and finally 650 mg. were given in the form of 50 cc. of a 10 per cent calcium lactate solution by stomach tube daily for 17 days. The dog received 2 units of parathormone for the first 7 days, 4 units for 5 days, the serum calcium rising to 14.8 mg. per 100 cc. on the 12th day of the experiment. Parathormone was discontinued for 7 days and was again begun on the 19th day of the experiment at 2 units daily. This dose was continued for 6 days, followed by 4 units daily for 4 days, 6 units daily for 6 days, and 10 units daily for 7 days. The serum calcium on the 27th, 33rd and 41st days was 12.2, 11.2 and 10.6 mg. per 100 cc., respectively. There was a progressive drop in spite of increased parathormone dosage, which was probably due to the fact that the dog was getting only about 100 mg. calcium daily during this period. The last serum calcium value was obtained just before the calcium supplement was increased to 1200 mg. daily on the 41st day. On the 43rd day, the dog still being on 10 units of parathormone daily, the serum calcium rose to 15.3 mg. per 100 cc. The parathormone was discontinued for 4 days. Treatment was resumed on the 47th day, with 6 units daily. The calcium intake was reduced

to 650 mg. by stomach tube. On the 51st day of the experiment serum calcium of 19.0 mg. per 100 cc. was found, and 18.5 mg. on the following day. On continuing the treatment, the serum calcium dropped to 12.6 mg. by the 58th day and on the 61st day it was 11.3 mg. The dog showed pronounced loss of appetite on the 57th day and left almost all of its food from the 62nd day on. It died on the 65th day. 2 hours before death the serum calcium was 15.8 mg. and the serum phosphorus 11.9 mg. per 100 cc. (The coincidence of these high values is a common observation in fatal hyperparathyroidism.)

At autopsy the external parathyroids seemed to be slightly smaller than usual. There were patches of pneumonia in the left lower, and right lower and middle lobes of the lung. The heart muscles were pale and oedematous. The kidneys were yellowish, the markings indistinct, and throughout the kidney white specks, apparently calcium deposits, were seen. There was an intense gastro-enteritis. The stomach wall was thickened to about five times normal. The stomach mucosa was extremely injected, and showed numerous mottled light-yellow areas, apparently containing calcium deposits. The injection of the intestines extended to the end of the ileum; the sigmoid and colon were free. The bones were softened, and broke easily. There were no swellings at the costochondral junctions.

The ribs on histological examination showed marrow fibrosis, but some lymphoid marrow was still present. The cortical bone was resorbed, and there were numerous Howship's lacunae and osteoclasts. Endochondral ossification was still in progress, the new formed trabeculae being lined by osteoblasts. Sections of the long tubular bones also showed marrow fibrosis and bone resorption, but to a lesser degree than the ribs. In ground-disk sections from the middle of the diaphysis enlarged vessel canals were found. The lungs were consolidated and calcium deposits were present in the inflammatory exudate. The stomach and intestines were congested, necrotic in places, and calcium deposits were found in the mucosa. The kidney tubules contained calcium casts. The parathyroid sections showed involutional atrophy of the parenchymatous cells.

Summary.—On a moderate calcium intake parathormone dosage was increased rapidly without adverse effects. When the calcium intake was increased, the serum calcium rose at first, but soon declined to normal values. However, the dog was apparently in a state of severe hyperparathyroidism for about 1 week before its death while it showed normal serum calcium values. There was terminal hypercalcemia and hyperphosphatemia. At autopsy the severe effects of acute hyperparathyroidism were observed in the soft tissues, while the bones showed the effects of chronic hyperparathyoidization. It is noteworthy that osteoid tissue formation was not prominent.

Dog 7.—Initial weight, 1.5 kg. Killed accidentally 106 days later, when it weighed 4.35 kg. The diet was supplemented with 1400 mg. of calcium in the form

of bone meal for the first 5 days. During the next 64 days 600 mg. of calcium (bone meal and calcium lactate) were added daily. Following that, for 37 days the dog received daily 50 cc. of 10 per cent calcium lactate by stomach tube (equivalent to 650 mg. of calcium), and was killed accidentally on the 106th day of the experiment by suffocation when the stomach tube was inserted in the trachea. Two units of parathormone were injected daily for the first 7 days, then 4 units for 17 days, 8 units for 4 days. The serum calcium values were 11.9 on the 12th day, and 11.3 on the 28th. Parathormone was increased to 12 units for 2 days, but was discontinued for the next 4 days because anorexia developed; no serum calcium determination was made. Before parathormone injections were resumed, serum calcium was 11.4 mg. per 100 cc. (on the 34th day). It rose to 15.6 mg. on the 39th day of the experiment, after parathormone had been given for 4 days (10 units daily). This was the first time hypercalcemia was observed in this dog, although signs of hyperparathyroidism had been present (anorexia). Parathormone was discontinued for 2 days, and then resumed, 8 units being given daily for 21 days until the 61st day of the experiment. The serum calcium was 12.2 mg. per 100 cc. on the 42nd day, 12.6 mg. on the 45th, 13.7 mg. on the 53rd, and 11.1 mg. on the 60th day of the experiment. Parathormone injections were increased to 14 units daily on the 62nd day. On the 63rd day the serum calcium rose to 14.5 mg. On the 67th day the serum calcium was 17.1 mg. but serum phosphorus was not raised. The animal refused food though he was active. Parathormone was discontinued for 2 days, and then resumed at 6 units daily. After 8 days it was increased to 8 units daily, which dose was continued from the 77th day to the 106th day. The serum calcium showed a regular drop from 11.5 to 9.8 between the 74th and 97th day of the experiment, in spite of the daily parathormone injections.

The animal at autopsy was well nourished. There was considerable abdominal fat. The soft tissues showed no gross pathological lesions. The dog was stunted in growth, the bones were short, and the cortex of the long tubular bones was thin. The ribs showed fibrosis of the marrow. The lymphoid marrow had been largely replaced by loose connective tissue containing numerous eosinophiles and a sprinkling of marrow cells. The blood vessels were dilated. Near the costochondral junctions and underneath the periosteum there were numerous osteoclasts with evidence of active osteoclastic resorption. A few small cysts were seen throughout the marrow. These were lined by flattened endothelioid cells and probably represented blood vessels dilated as a result of circulatory stasis. The haversian spaces of the compacta were enlarged, but in spite of the fibrosis of the marrow and the resorption of the bone most of the trabeculae were lined by osteoblasts and active endochondral ossification was in progress. There was metaplastic new-bone formation. Sections of the long tubular bones showed essentially the same changes as the ribs, but in somewhat lesser degree. The ground disks of sections from the diaphysis of the long tubular bones also showed resorption, the vessel canals being much enlarged. The parathyroid showed involutionary changes and metastatic calcification of the kidney tubules and stomach mucosa was found.

Summary.—On parathormone therapy with an intake of at least 600 mg. of calcium daily there was bone resorption and marrow fibrosis, but there was also new-bone formation. The bone was deposited on cartilaginous trabeculae in the course of endochondral ossification. Having succeeded in maintaining this dog for a longer period than No. 6, we obtained a more severe degree of bone resorption and marrow and bone fibrosis.

Dog 8.—Initial weight, 1.1 kg. Killed to terminate the experiment 168 days later, at which time it weighed 2.9 kg. No supplementary calcium was given except between the 52nd and 60th days, when the dog received 650 mg. of calcium daily in the form of calcium lactate by stomach tube.

This dog received at first 2 units of parathormone daily; from the 8th to the 24th day, 4 units daily; from the 25th to the 29th day, 6 units daily; from the 30th to the 34th day, 12 units daily; from the 35th to the 39th day, 16 units daily; from the 40th to the 48th day, 6 units daily, and from the 49th to the 51st day, 10 units daily. Between the 52nd and the 107th day parathormone was discontinued. On the 108th and 109th days 3 units were given daily; then 8 units from the 110th to the 158th day, and 20 units daily to the 168th day, when the dog was killed to terminate the experiment.

Blood calcium studies showed strikingly the progressive fall that occurred as the animal was decalcified. The serum calcium was 13.0 mg. per 100 cc. on the 12th day, 11.4 on the 28th, 10.9 on the 34th, 9.6 on the 42nd, and 8.1 on the 49th day. The dog at this time was rigid, dull, and had respiratory difficulties. On the 53rd day it had tremors and tetany, the serum calcium being 7.8 mg. per 100 cc. The tetanic symptoms may be taken as an indication of extreme exhaustion of calcium from the bones and soft tissues. At this time the forelegs were bowed outward, and the animal was weak and had difficulties in walking. It was decided to discontinue the parathormone and administer 650 mg. of calcium daily in order to preserve the animal's life. Between the 53rd and 60th day, when the dog was receiving the large calcium supplement, its clinical condition improved rapidly, the serum calcium rising to 9.0 mg. On the 60th day the calcium was discontinued and the serum calcium began to show a consistent drop reaching 7.8 mg. per 100 cc. by the 97th day. On the 108th day parathormone was again started at 8 units daily. The serum calcium remained fairly constant at about 8.0 mg. per 100 cc. or less. On the 168th day, when the experiment was terminated, and after the dog had been on 20 units for 10 days, the serum calcium was 7.8 and the serum phosphorus 6.4 mg. per 100 cc., although at first the animal had responded to the increased dose by a serum calcium as high as 10.4 mg.

The clinical condition of the dog makes an interesting study. On the 40th day its abdomen was distended, apparently as a result of hyperparathyroid hypotonia. Between the 48th and 53rd days there was difficulty in breathing, dullness and rigidity, with frequent tetanic seizures with spasmodic defecation and urina-

tion when the dog was handled. On the 53rd day respiratory failure developed during a tetanic seizure. At this time a serum phosphorus value of 10.7 mg. per 100 cc. was found. Artificial respiration restored the dog in several minutes, after which the muscles were relaxed. High phosphorus values were observed frequently, particularly after tetanic seizures, which varied in severity during the period that followed, which lasted for about 8 weeks, while the dog was not receiving any parathormone. There was rapid improvement after calcium therapy was initiated. The appetite improved, and the animal walked about the cage. The calcium was discontinued after about a week, but parathormone therapy was not resumed for 7 weeks longer, until the 107th day. The tremors reappeared together with spasmodic defecation, grunting respiration, and atony of the abdominal wall. Greater deformities of the forelegs developed, the plantigrade stance becoming worse. Shortly before the dog was killed, bloody stools and bloody diarrhea appeared, probably due to parathormone overdosage (without hypercalcemia).

Weight at autopsy was 2.9 kg. The dog had attained its maximum weight of 3.58 kg. 3 weeks previously. The animal had been greatly stunted, both humeri were rotated on their long axes, as observed in osteomalacia, and even on gross examination marked softening of the bones was evident. The internal organs showed nothing ususual in the gross.

On histological examination the ribs showed very extensive replacement of the marrow by cellular connective tissue. Embedded in it were a few islands of lymphoid marrow. In these islands the blood forming cells were for the most part well preserved, and red and white blood cells were maturating. The megakaryocytes were few and degenerated. The spindle connective tissue replacing the marrow was in places oedematous, and it contained a sprinkling of lymphocytes, polymorphonuclear leucocytes, some macrophages and plasma cells. A few lymphoid marrow cells could still be observed scattered through the connective tissue, which was quite vascular. Some osteoclasts were also observed in the marrow connective tissue. Very little remained of the original cortical bone, which had been resorbed, as evidenced by the enlarged vessel canals filled with fibrous tissue. One of the ribs examined showed a fracture through the cortex, and there was an attempt at repair by the formation of subperiosteal and endosteal callus. Throughout the fibrosed marrow a large number of osteoid and lamellar-bone trabeculae were observed. Many of these osteoid trabeculae were covered by osteoclasts, while the lamellar bone trabeculae were generally naked, and showed osteoclasts in considerable numbers in or near Howship's lacunae. At the costochondral junctions there was considerable osteoid-bone, and the intertrabecular connective tissue contained numerous osteoclasts. Many small cyst-like spaces were observed throughout the scarred marrow, but were most numerous at the costochondral junctions. They were lined by very flat endothelioid cells, and many were surrounded by lymphoid marrow. Some of these cysts were definitely blood-vascular spaces, containing red blood cells. These spaces were apparently related to stasis of marrow circulation. Some were possibly of

lymphatic origin. However, it seems to be impossible to trace definitely the origin of all of the cyst-like spaces.

Examination of longitudinal sections of the upper end of the humerus showed that the articular cartilage was normal. A very striking finding was the preservation of the marrow of the epiphysis, which was cellular and showed no fibrosis. The bone trabeculae of the epiphyses were thin, but not transformed. The epiphyses were therefore entirely free of ostitis fibrosa. The metaphyses and diaphyses, however, showed cortical resorption, osteoid-bone formation, numerous osteoclasts in Howship's lacunae, fibrosis and marked congestion of the marrow and enlargement of the vessels with marrow hemorrhage. There had been a pathological fracture of the humerus. The femur showed similar changes, and even to a greater degree. They were confined to the metaphysis and diaphysis. The femur also had a pathological fracture. The sections of several other tubular bones showed similar changes, but to a lesser degree.

Cross-sections from the diaphyses of the long tubular bones showed extremely enlarged vessel canals containing fibrous marrow. Some Howship's lacunae and osteoclasts were also seen. The haversian canals were lined by osteoblasts. Ground disks from the same regions also showed marked resorption.

The kidneys on the whole were well preserved, showing in places nests of lymphoid cells, a few sclerosed glomeruli, and calcium in some tubules. Numerous liver cells were replaced by fat. The other tissues showed nothing unusual. The specimens were not fixed or stained specially for calcium.

Summary.—This dog showed extensive resorption and fibrous replacement of the bone and marrow with considerable new bone formation (osteoid tissue). The fibrosed marrow was congested and contained cyst-like spaces. Deformities of the legs developed and histologically there was evidence of numerous pathological fractures. In spite of many evidences of severe hyperparathyroidism, the serum calcium showed consistent decline, apparently accompanied by lowered tissue calcium, leading eventually to tetany. The development of all of these consequences of hyperparathyroidism was no doubt enhanced by the calcium-poor diet, but cannot be attributed, of course, to the diet as such.

Dog 9.—Initial weight, 2.25 kg. The dog gained weight slowly but continously to the termination of the experiment, 167 days later, when it weighted 5.35 kg. Calcium supplement, 200 mg. daily for the first 23 days (bone meal and calcium lactate), and 1300 mg. from the 24th to 27th day (calcium lactate). From the 28th to the 88th day the dog received daily 650 mg. of calcium (50 cc. of a 10 per cent solution of calcium lactate by stomach tube). Between the 89th and the 143rd day no supplementary calcium was given, then 100 mg. of calcium (bone meal and calcium lactate) were added and continued to the end of the experiment.

Four units of parathormone were injected during the first 6 days, and 8 units from the 7th to the 10th day. The serum calcium was 16.4 mg. per 100 cc. on the 10th day. Parathormone was omitted from the 11th to the 16th day, the serum calcium falling to 11.3 mg. on the latter day. From the 17th to the 20th day 6 units were given daily and the serum calcium rose to 12.4 mg. On the 21st and 22nd days the dog received no parathormone; on the 23rd and 24th, 8 units daily; none on the 25th and 26th. The serum calcium was 11.4 mg. per 100 cc. on the 24th and 11.0 on the 27th. From the 27th to the 43rd day the dog received 8 units daily; the serum calcium rising to 15.0 mg. on the 35th day and 15.5 on the 36th. The treatment being continued, serum calcium fell to 10.9 mg. on the 42nd day. On the 44th day the parathormone was increased to 12 units daily. The serum calcium was 12.1 mg. on the 45th day. On the 51st day, after some loss of appetite, the parathormone was decreased to 6 units daily, raised to 8 units daily on the 59th day, and continued at this dose to the 138th day. The serum calcium remained constant at normal values while the animal received 650 mg. of calcium daily (to the 88th day). During the period when no calcium supplement was given (88th day to 143rd day) the serum calcium dropped consistently. It was 10.0 mg. per 100 cc. on the 100th, 9.6 on the 103rd, 9.6 on the 112th, 8.5 on the 133rd, and 8.8 on the 140th day. On the 140th day the parathormone was raised to 20 units a day and continued at this dose until the termination of the experiment of the 167th day. The serum calcium rose to 9.9 mg. per 100 cc. on the 147th day, and was 10.1 on the 150th, 10.8 on the 154th day, 10.7 on the 161st day, and 10.6 on the 167th day.

Clinically this animal showed hypercalcemia and hyperparathyroidism on several occasions. The appetite was generally good and there was a progressive gain in weight. There was marked stunting of growth, the forelegs were bowed outwards, the bones were thin. The dog was well nourished at autopsy and otherwise showed nothing unusual in the gross.

On histological examination, the ribs showed extensive fibrous replacement of the marrow, but considerable lymphoid marrow still remained. The fibrous tissue of the marrow was oedematous. Marked resorption of the existing bone was evident, and numerous osteoclasts were observed in Howship's lacunae in the marrow connective tissue. Numerous trabeculae of osteoid-bone were observed near the costochondral junction. Some cyst-like spaces were present. The changes were of the same order, but somewhat less pronounced than in Dog 8. The long tubular bones also showed resorption of the compacta, enlargement of the vessel canals, which were filled with fibrous tissue. There was subepiphyseal cartilage osteoid-bone formation, and some fibrosis of the marrow. The epiphyses were free of these changes. Stained cross-sections and ground disks made of bone taken from the middle of the diaphyses of tubular bones showed enlarged vessel canals, and subperiosteal and subendosteal resorption. In the stained sections numerous osteoclasts were seen in the enlarged vessel canals, as well as some fibrous tissue and osteoid.

In a section of the lower jaw bone resorption, fibrosis, osteoclasts and cyst-like spaces were found, while a section from the calvarium showed similar changes and some osteoid-bone formation.

Nothing unusual was observed in the kidney, and no calcium deposits were found in sections stained by the v. Kossa method. None of the other soft tissues showed anything striking, and calcium deposits were found only in the stomach. The parathyroid glands seemed to have undergone simple involutional atrophy.

Summary.—This dog also showed the typical bone changes of chronic hyperparathyroidism. The histological picture satisfies the criteria established as necessary for the diagnosis of ostitis fibrosa. The absence of metastatic calcification in the tissues of this dog, in spite of the prolonged treatment, is significant. It seems to indicate that while metastatic calcifications are to be expected in acute hyperparathyroidism with hypercalcemia, the absorption of the calcium deposits is likewise to be expected in the stage of chronic hyperparathyroidism without hypercalcemia. (See also Dog 10.)

Dog 10—Initial weight, 1.05 kg. Weight at termination of the experiment, 185 days later, 4.8 kg. Greatest weight, 5.3 kg., was attained 19 days before the dog was killed. Calcium supplement, 560 mg. calcium daily for the first 5 days (bone meal and 200 mg. calcium daily from the 6th to the 105th day (bone meal and calcium lactate). Calcium was discontinued until the 162nd day, when 100 mg. of calcium daily was added and continued to the 185th day. The dog was injected with 2 units of parathormone daily for the first 7 days, 4 units daily to the 11th day, at which time the serum calcium was 15.2 mg. per 100 cc. From the 12th to the 24th day the dose was reduced to 2 units daily. The serum calcium on the 28th day was 12.7 mg. On the 30th day parathormone was increased to 8 units daily, but injections were discontinued from the 33rd to the 40th day because of loss of appetite. They were resumed on the 41st day at 6 units daily. The serum calcium was 14.7 mg. on the 42nd day, 12.5 on the 45th, 12.3 on the 53rd, and 11.5 mg. on the 60th day. On the 61st day parathormone was increased to 12 units daily. The serum calcium rose to 12.2 mg. on the 63rd, and 13.0 mg. on the 67th day, with associated loss of appetite. After omitting one dose of parathormone, it was decreased on the 69th day to 6 units daily until the 78th day, at which time the serum calcium was 11.6 mg. On the 79th day the injection of 8 units of parathormone was begun and this dose was continued to the 157th day. Serum calcium was 11.5 mg. on the 81st day, 10.7 on the 88th, and 11.6 on the 96th day. On the 106th day the supplementary calcium was discontinued, the parathormone dose being maintained at 8 units daily. The serum calcium was 10.8 mg. on the 118th day, 12.5 on the 112th, 10.7 on the 130th, 11.1 on the 151st, and 10.8 on the 158th day. On the 160th day the dose of parathormone

was increased to 20 units daily, but the serum calcium remained unchanged at 11 mg. on the 165th day, 11.2 on the 168th, 11.0 on the 172nd, 11.2 on the 179th, and 11.2 on the 185th day, when the dog was killed to terminate the experiment.

On autopsy the dog was well nourished. Otherwise the autopsy showed nothing unusual in the gross, except that there was slight bowing of the bones. The dog was markedly stunted. The changes in the bones of this dog were essentially the same as in No. 9. Histological examination of the soft tissues revealed nothing unusual. The splenic pulp was somewhat fibrosed and some of the liver cells were fatty. Tissues appropriately fixed and stained showed a striking absence of calcium. This was in significant contrast with our observations of metastatic calcifications in dogs dying of acute hyperparathyroidism. Only a few kidney tubules showed some calcium in the lumina.

Summary.—The tissue changes and their significance were the same as in Dog 9. Bone lesions characteristic of ostitis fibrosa were produced as a result of prolonged treatment with parathormone.

DISCUSSION

We have produced ostitis fibrosa in young growing puppies. We believe that the period of active skeletal growth is more suitable for the development of this condition. During the period of growth the demand for calcium is great, but even on a liberal calcium intake parathormone interferes with calcium utilization, and with the formation of normal bone, the calcium balance being negative on appropriate dosage. An analogy is found in the well-known requirement in the standardized procedure for the production of experimental rickets, the development of which is favored by rapid growth. The changes that we have observed in experimental hyperparathyroidism are not of the type that are caused by low calcium diets. On such diets animals are not stunted, provided the diets are not deficient in vitamins; no marrow destruction and fibrosis are found. These lesions seem to be specific effects of parathormone treatment. The decalcification in hyperparathyroidism is not only more rapid than in simple calcium deprivation, but perhaps because of the greater rate of decalcification the secondary effects develop which serve to distinguish the two conditions.

That parathormone affects the lymphoid marrow cells directly, in addition to its effects on the bone, was observed in all dogs dying of acute hyperparathyroidism. We believe that during acute hyper-

parathyroidism destructive changes appear also in other lymphoid tissues. There seems to be no doubt that the severe marrow fibrosis is in part an expression of the healing of the injured marrow during prolonged parathormone therapy.

It may well be that the immediate antecedent of experimental ostitis fibrosa is a condition, perhaps related to the disturbed acid-base equilibrium, which may also be caused by other agents than parathormone. However, it seems essential that the action be continuous, and that the condition which it causes be maintained for long periods without endangering the life of the experimental animal, or a certain minimum of well-being. Parathormone is specific in the sense that it satisfies these requirements.

In acute experimental hyperparathyroidism, metastatic calcifications are generally observed, associated with hypercalcemia. We have confirmed this observation. In some of our animals, the stage of acute hyperparathyroidism was succeeded by chronic hyperparathyroidism, without hypercalcemia. In these animals, when they came to autopsy metastatic calcifications were absent or nearly absent. We suggest that metastatic calcifications had been present in these animals earlier, and were resorbed during the chronic stage of hyperparathyroidism.

It has been widely realized that parathormone effects are to be judged by calcium excretion, particularly in the urine, rather than by a rise of serum calcium. And yet, absence of parathormone effects has been frequently inferred from the absence of hypercalcemia. It therefore remains necessary to emphasize that parathormone effects on bone may be observed, as our protocols prove, even when the calcium values are normal or lower than normal.

The lesions produced in these young animals were of the order observed in v. Recklinghausen's disease (ostitis fibrosa cystica, or the hypostotic-porotic form of osteodystrophia fibrosa). It is well known that clinically most of the cases of ostitis fibrosa cystica of v. Recklinghausen come to notice before middle age, the condition probably having developed for many years without clinically prominent symptoms. Hirsch, basing his conclusions on X-ray experience, believes that most of these cases begin early in life (50).

One of the very striking features observed in our young dogs whose

epiphyses were as yet not fused with the diaphyses, and in whom epiphyseal cartilage plates were still present, was the absence of fibrous osteodystrophy in the epiphyses. The epiphyses at most showed some simple bone atrophy, but marrow fibrosis and active bone resorption, with Howship's lacunae and osteoclasts, were absent. The lesions were most prominent in the metaphyses, where active new bone formation would normally take place. It seems quite definite that bone injury and marrow fibrosis observed by us in these experiments are not dependent upon vascular changes, as the epiphyses are also exceedingly vascular during the growing stage and would share in the changes if the vascular factor were decisive.

The presence of osteoid tissue as a requisite for establishing the diagnosis of ostitis fibrosa seems to have been given too much significance. In advanced clinical cases with considerable deformity, it appears almost always. Its appearance, we believe, is related to the physiological need of reinforcing bones subject to decalcification. In our dogs we observed osteoid tissue formation when decalcification was most severe, and when there was definite need for repair, as for instance in response to a gross fracture. We conceive that rapid decalcification, when gross fractures do not occur, leads to a weakening of the bone at innumerable points, especially if the animal is active. In view of the stresses and strains exerted at these points, microscopical fractures may be conceived, in the repair of which osteoid is produced. Osteoid may also be formed on greatly thinned bone as an expression of the need to repair.

In regard to the presence of cysts in our dogs with advanced fibrous lesions, these are, we believe, and as many pathologists have observed in clinical material, due for the most part to circulatory stasis that results from the distortion and scarring of the marrow incident to the development of the ostitis fibrosa.

In dogs dying of acute hyperparathyroidism we observed reduction in the size of the external parathyroids to about one-half their usual size. Microscopically, the reduction in size was found related to a diminution in the size of the parenchymatous cells and to their distortion. We interpret this picture as evidence of functional involutional atrophy due to substitution therapy.

SUMMARY

These experiments have shown that parathyroid extract (parathormone Collip) can be injected into puppies in increasing amounts for long periods without fatal results. Thus time is allowed for bone changes to develop. Long continued injection leads to progressive decalcification and resorption of the existing bone, to fibrous replacement of the marrow, and to the production of the other features characteristic of ostitis fibrosa. Deformities eventually appear. It is safe to assume that the bone changes produced by hyperparathyroidization have the same pathogenesis as those observed in clinical cases believed to be instances of hyperparathyroidism—that is, cases with a negative mineral balance and decalcification of the skeleton.

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EXPLANATION OF PLATES

PLATE 35

FIG. 1. Rib of Dog 9 showing costochondral junction. The marrow is fibrosed, some islands of lymphoid marrow are still present. A few cysts are observed. Magnification 18×. Stained with hematoxylin and eosin.

FIG. 2. Rib of Dog 10. The marrow is fibrosed and contains numerous osteoclasts. Cysts are present. Magnification 20×. Stained with hematoxylin and eosin.

FIG. 3. Rib of Dog 8. The marrow is fibrosed and numerous cysts are seen. Considerable newly formed trabeculae are present. In places there is disappear-

ance of the cortical bone. Magnification 10×. Stained with hematoxylin and eosin.

FIG. 4. Rib of Dog 8 showing remaining islands of lymphoid marrow, many of which surround cysts. Fibrosis of the marrow, thinning and transformation of the cortex are more evident than in Fig. 3. Magnification 20×. Stained with hematoxylin and eosin.

FIG. 5. A higher power picture from same rib showing marrow fibrosis, cysts, and newly formed fiber-bone. Magnification 150×. Stained with hematoxylin and eosin.

FIG. 6. Shows part of the epiphysis (above) and metaphysis (below) the epiphyseal cartilage plate of femur of Dog 8. There is no endochondral-bone formation at the epiphyseal cartilage plate. Lymphoid marrow is observed in the epiphysis, where marrow fibrosis is absent. In the metaphysis marrow fibrosis and numerous cysts are seen. Magnification 16×. Stained with hematoxylin and eosin.

PLATE 36

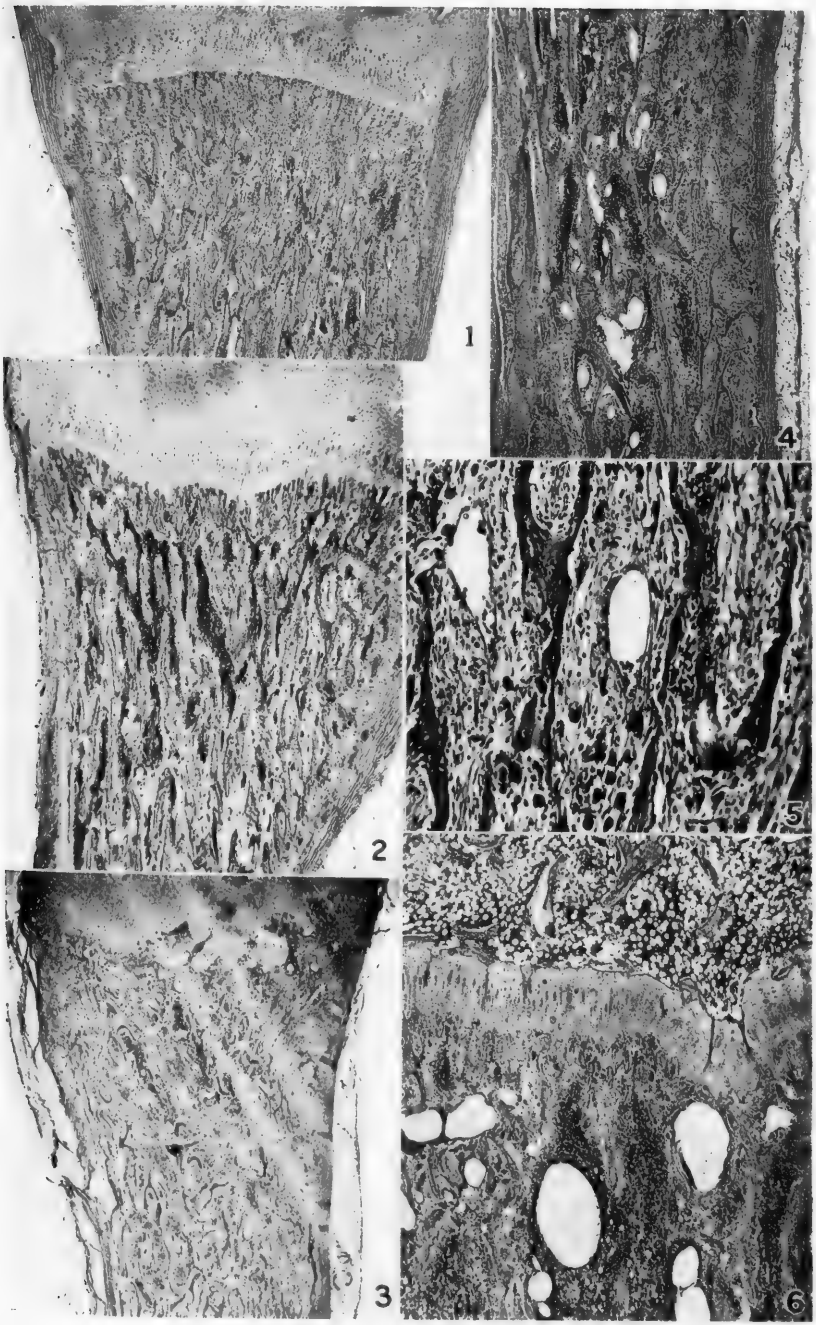
FIG. 7. The diaphysis of the femur of Dog 8 showing considerable marrow fibrosis with resorption of bone. Magnification 20×. Stained with hematoxylin and eosin.

FIG. 8. Osteoid tissue in the fibrosed marrow of the femur of Dog 10. Magnification 60×. Stained with hematoxylin and eosin.

FIG. 9. Fiber-bone formation in the fibrosed marrow. Magnification 300×. Stained with hematoxylin and eosin.

FIG. 10. Shows extensive osteoclastic resorption below the epiphyseal cartilage plate of the humerus of Dog 9. Magnification 85×. Stained with hematoxylin and eosin.

FIG. 11. Shows resorption and fibrosis of bone from calvarium of Dog 10. Magnification 85×. Stained with hematoxylin and eosin.



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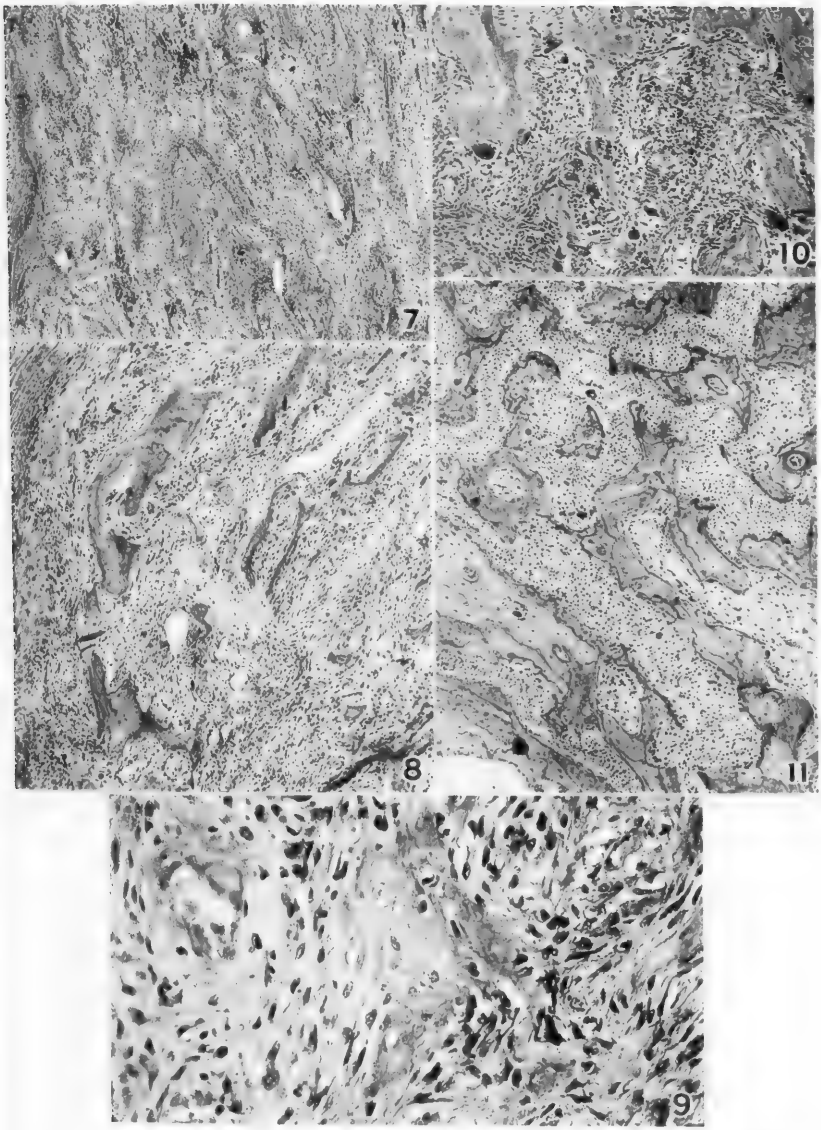

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A COMPARISON OF THE CHEMICAL ALTERATIONS IN THE BLOOD OF RATS INFECTED WITH PATHOGENIC AND NON-PATHOGENIC TRYPANOSOMES

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In a recent study (1) of some of the factors involved in acute infections with *Trypanosoma equiperdum* in rats it was found that the CO_2 capacity of the serum was lowered, the lipoid phosphorus, and lecithin, increased, and the liver glycogen greatly diminished or absent. The blood sugar, on the other hand, remained at a normal level until late in the disease. Cholesterol remained unchanged in amount, while the non-protein nitrogen and uric acid increased only subterminally with the partial breakdown of the kidneys.

It seemed of interest to investigate the factors which had proved to be influenced in the pathogenic infection, in infections due to the non-pathogenic *Trypansoma lewisi*, to determine a possible injurious effect of this parasite. The blood sugar in rats infected with this organism has already been reported upon *in extenso* (2), and found to remain normal.

The methods are those that were used in the previous work. The results for the lipoid phosphorus, the calculated lecithin, and the liver glycogen, are given in Table I. Table II summarizes the experiments for the determination of the CO_2 capacity, and Table III contains a comparison between the fatal and the non-fatal infections, in respect to these three factors.

The data for the 21 animals in Table I are arranged to illustrate the rise and fall in number of the trypanosomes throughout the infection. The determinations were made from animals with increasing numbers of trypanosomes up to 450,000 per cubic millimeter (No. 11): the remaining animals were those in which the crisis was passed. The lipoid phosphorus was determined, and the lecithin calculated,

in normal animals before the infection was given, and again when the infection had reached the stage indicated by the trypanosome counts. The percentage of variation between the two determinations is also given.

This variation is positive in 9 cases and negative in 4. The normal range for lecithin, based on the results of this and the previous paper, seems to lie in the rat between about 170 and 250 mg. per 100 cc. of blood. In 4 of the animals only a single determination was made, and all of these lie within the normal range (Nos. 5, 12, 14, and 19). In one animal (Rat 6) the upper limit is transcended, and the variations throughout, both positive and negative, are relatively slight and without the marked upward trend which is apparent in the infections with *T. equiperdum*. These figures for rats are slightly higher than those for human blood as determined with this method by Rosen and Krasnow (3); they found the range to be from 160 to 240 mg.

The figures showing the amount of liver glycogen, calculated as glucose per gram of liver, are also included in Table I. This arrangement does not imply any connection between the two factors. Glycogen is found to be present in significant amounts in every case, and does not vary with the rise and fall of the parasites. This finding is in contrast with the results of Regendanz and Tropp (4). These workers have published figures for 4 rats infected with *T. lewisi*, which show, they believe, that the glycogen is significantly lowered. Their animals had been splenectomized in order to increase the number of organisms in the blood. It is impossible to tell, however, to what extent such an increase has actually occurred, since they do not publish any trypanosome counts; nor are their figures for the glycogen content much lower than "normal," especially in view of the great variations to which this constituent is subject.

Table II presents the CO_2 capacities of the serum of 12 infected rats. All of these animals were taken for study at the time when the parasites were in the stage of rapid increase. The values found are all within the normal range. They show that the rats do not suffer from an acidosis during the infection, although acidosis is present from an early stage in infections with *T. equiperdum*.

TABLE I

Lipoid Phosphorus, Lecithin, and Liver Glycogen, as Glucose, in Rats Infected with Trypanosoma lewisi

Rat No.	Before infection		After infection		Percentage variation	No. trs. per cu.mm.	Glucose. Mg. per gram of liver
	Lipoid p. Mg. per 100 cc. blood	Lecithin. Mg. per 100 cc. blood	Lipoid p. Mg. per 100 cc. blood	Lecithin. Mg. per 100 cc. blood			
1						50,000	12.3
2						75,000	12.0
3	8.9	222.5	10.1	252.5	+13	92,500	
4	8.1	202.5	8.5	212.5	+5	125,000	17.6
5			8.65	216.2		140,000	13.3
6	9.85	246.2	11.6	290.0	+18	150,000	8.1
7	8.25	206.2	10.0	250.0	+21	167,000	8.0
8						200,000	18.0
9	8.6	215.0	9.9	247.5	+15	317,500	7.1
10	9.05	226.2	9.9	247.5	+9	342,000	4.8
11	9.1	227.5	9.0	225.0	−1	450,000	9.8
12			9.5	237.5		200,000	11.0
13	8.9	222.5	8.65	216.2	−3	130,000	35.0
14			8.3	207.5		125,000	9.5
15	8.6	215.0	8.3	207.5	−3	105,000	6.6
16	9.2	230.0	9.3	232.5	+1	100,000	20.4
17	9.15	228.7	8.6	215.0	−6	100,000	
18						100,000	15.5
19			9.05	226.2		62,500	6.1
20	8.8	220.0	9.8	245.0	+11	62,500	
21	7.35	183.7	7.75	193.7	+5	25,000	

TABLE II

Carbon Dioxide Capacity of Serum in Rats Infected with Trypanosoma lewisi

Rat No.	Trypanosomes per cu.mm.	CO_2 capacity. Vol. per cent
1	100,000	56.0
2	137,000	56.0
3	150,000	64.6
4	175,000	71.1
5	180,000	60.7
6	200,000	63.6
7	200,000	69.1
8	225,000	68.3
9	230,000	69.1
10	237,500	62.6
11	250,000	58.8
12	275,000	64.4

DISCUSSION

Table III gives a comparison of the pathogenic and non-pathogenic infections. In *T. lewisi* rats the lecithin varies from the normal −6 per cent to +21 per cent, and shows an average increase of +6.5 per

TABLE III

Percentage Variations of Lecithin, and Average Liver Glycogen Content, as Glucose, throughout Infections with Trypanosoma lewisi and Trypanosoma equiperdum

	Lecithin	Glucose. Mg. per gram of liver
T. lewisi	−6 per cent to +21 per cent avg. + 6.5 per cent (9 increases, 4 decreases)	12.6 mg. (no negative cases)
T. equiperdum	+8 per cent to +99 per cent avg. 35.6 per cent (11 increases, no decreases)	1.8 mg. (7 negative cases out of 11)

Variation in CO_2 Combining Capacity at Various Concentrations of Trypanosoma lewisi and Trypanosoma equiperdum per Cubic Millimeter of Blood

	No. trypanosomes	CO_2 combining cap. Vol. per cent
T. lewisi	137,500	56.0
T. equiperdum	137,500	37.0
T. lewisi	200,000	69.1
T. equiperdum	200,000	13.6
T. lewisi	237,500	62.6
T. equiperdum	225,000	45.0
T. lewisi	275,000	64.4
T. equiperdum	275,000	35.0

cent; 9 of the animals showed increases and 4, decreases. With *T. equiperdum* all the animals showed increases, ranging from +8 per cent to +99 per cent, and averaging +35.6 per cent. The same difference is shown in the comparison of the glycogen. With *T. lewisi* this constituent was present to an average extent of 12.6 mg. of glucose per gram of liver, and no animals were found in which it could

not be demonstrated; in the livers of *T. equiperdum* animals, on the other hand, it was absent in 7 out of the 11 cases, and averaged for the series 1.8 mg. per gram of liver. The CO_2 capacities are contrasted in 4 sets of animals in which the concentrations of trypanosomes per cubic millimeter were similar. In each case the *T. lewisi* animals show a normal, and the *T. equiperdum* animals an abnormal, capacity.

In general, the table, together with the others in this and the previous paper, shows that the action of *T. equiperdum* on these three factors is not due to the influence of its numbers, since equal numbers of *T. lewisi* are without effect, but to some action which it has upon the host. To apply the term "toxin" to this effect is simply a begging of the question, since no completely acceptable evidence for the existence of such a substance has ever been adduced.

The possibility must be kept in mind that counting the trypanosomes in the peripheral blood may not be a valid method of comparing the extent of the two infections, since large numbers of the pathogenic organisms might be retained in, and have their effect upon, the inner organs. Work now in progress shows that such an "inner action" does occur when guinea-pigs are infected with *T. equiperdum*, in that a severe anemia appears in these animals relatively long before the parasites are demonstrable in the blood stream, or while their concentration is extremely low.

By the study of the factors here discussed it should be possible to determine whether "pathogenic" strains of *T. lewisi* exist. At present, the evidence for the pathogenicity of this organism is not convincing; especially is this so since Marmorston-Gottesman and Perla (5) have shown that death of normal animals infected with *T. lewisi* may really be due to a concomitant infection with *Bartonella muris*.

SUMMARY

Blood samples from rats infected with *Trypanosoma lewisi* give normal values for lipoid phosphorus, lecithin, CO_2 combining capacity, and liver glycogen.

When these results are compared with the results of similar experiments with *T. equiperdum* infections, on the basis of the concentration

of trypanosomes in the blood, it is found that the pathogenicity of the latter organism does not depend upon its numbers as affecting the blood, but that it must be in some other way injurious to the host. Whether the injury is due to a true toxin, an endotoxin, or mechanical interference is not yet known.

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STUDIES IN THE COMMON COLD

IV. Experimental Transmission of the Common Cold to Anthropoid Apes and Human Beings by Means of a Filtrable Agent

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In this study of the upper respiratory infections usually grouped under the heading of common cold, our primary interest has been in etiology. In earlier work (1, 2), two groups of organisms were investigated for possible causative relationship: (a) the bacteria more readily cultivated from the upper respiratory tract and (b) the group of Gram-negative, filter-passing anaerobes first described by Olitsky and Gates (3) and Olitsky and McCartney (4) and more recently in studies by Branham (5), Noble and Brainard (6) and others.

In our work a number of normal individuals were taken and by serial cultures, over periods of several months, the basic bacterial flora of their noses and throats was determined. Concurrently detailed observations were made of quantitative and qualitative changes occurring in this flora in the course of colds. The bacteria included in the first study (1) were streptococcus, hemolytic and non-hemolytic; *B. pfeifferi*, including the hemolytic forms; Gram-negative cocci; diphtheroids; *Staphylococcus albus*, *aureus* and *citreus;* pneumococcus and other occasionally encountered miscellaneous organisms. In the second study (2) the various types of filter-passing anaerobes referred to above were included.

From the results obtained in this work we were led to conclude that none of the organisms in the first group are of primary etiological significance, although some of them are of importance as secondary invaders at times and that the filter-passing anaerobes constitute

part of the normal flora of the upper respiratory tract and do not seem to bear a causative relationship to colds.

Having failed in these attempts to assign a causative rôle to bacteria in these two groups, we were led logically to testing the hypothesis that colds may be initiated by a filtrable virus. The present report deals with an investigation of this possibility, using the medium of ape and of human transmission experiments.

In 1914 Kruse (7), later Foster (8) and more recently Olitsky and McCartney (4) reported successful transmission experiments using filtrates obtained from the nasal secretions of individuals suffering from colds. However, the following workers, Schmidt (9), Williams and her co-workers (10), Branham and Hall (11) and Robertson and Groves (12) failed in their efforts to confirm these results. In general the method used by these workers has been the intranasal inoculation of human volunteers.

In view of the difficulty of securing appropriate human subjects and particularly of effectively quarantining them, it was felt that animals would be far more satisfactory for transmission experiments. In searching about for such suitable animals it was learned that anthropoid apes were supposed to catch colds. Accordingly, curators of zoological gardens, and dealers and workers with these animals were sought out for confirmation of this report. It was found very soon that all workers with the higher apes were unanimously of the opinion that these animals readily caught colds from human beings similarly afflicted, and further that these colds were more or less similar clinically to those observed in man.

I. Suitability of Chimpanzees for Experimental Work in Upper Respiratory Infections

Having satisfied ourselves with respect to the probable suitability of the anthropoids for our purposes, we began acquiring chimpanzees. Young animals, aged 2 to 4 years and weighing from 15 to 30 pounds were chosen. In general, at these ages, the animals are quite gentle and with kindly training very rapidly become most cooperative. In the course of the first year's work we were able to collect suitable chimpanzees to the number of six. At the end of the first year the

colony numbered eight and during the last year two more animals have been added.

The animals have been kept in cages in rooms where a constant temperature of approximately 80°F. has been maintained by thermostatic control. They have been protected very carefully from contact with outsiders and all workers have been required to wear masks upon entering. When the animals have been used for transmission experiments they have been transferred from the stock room to special quarantine rooms where extreme precautions have been taken to exclude all possible infection from without. In general, everything entering the rooms including most of the food is sterilized. All workers wear sterile gowns, masks, caps and rubber gloves. That this system is quite effective has been shown by the fact that no accidental infection has ever occurred among the quarantined animals although there have been many such spontaneous infections among the animals in the stock room.

General Observations on Susceptibility of Chimpanzees to Colds.—Very early in our work we learned to our sorrow the truth of the reports regarding the susceptibility of the anthropoids to human upper respiratory infection. While our animal quarters were still in process of construction one of the first two chimpanzees acquired by us, while boarding at the animal dealer's, caught a cold from his keeper. The infection went over to what appeared to be a pneumonia and the animal finally succumbed. Post mortem examination showed bronchopneumonia and from the lungs there were recovered hemolytic streptococci, Pfeiffer's bacillus, *B. alkaligenes* and a Gram-negative anaerobic filter passer.

Since that time we have had abundant opportunity to observe the readiness with which these animals catch colds from human beings afflicted with these infections. When the men handling the animals have had colds the apes in the stock room frequently have contracted the infection even though the men have always worn masks. Colds of this type also spread rapidly from ape to ape. Such spontaneous infections have never occurred in the quarantine rooms as noted above, as in addition to the precautions already mentioned rigorous exclusion of workers with colds has been maintained.

When a chimpanzee catches a cold, the clinical picture presented is very similar to that observed in a human child.

At first there is a small amount of glairy mucus in the nostrils. By the end of the first day the animal usually appears quite sick, lassitude being fairly striking. The eyes are puffy and drooping, there is a moderate to profuse nasal discharge of thin mucus which runs down over the upper lip; there is definite nasal obstruction which makes it difficult for the animal to take liquid food and the breathing becomes audible. There is occasional sneezing and cough. The appetite is usually moderately impaired, rarely there has been diarrhea. Usually there is no elevation of temperature. By the second or third day the nasal discharge becomes mucopurulent. The throat at times has appeared inflamed. By the fourth or fifth day the animal is usually much better and the discharge and nasal obstruction become much less. Recovery is usually complete in a week to 10 days. Occasionally there is a persistence of cough for several days resembling the bronchitis so frequently noted as a complication of colds. Occasionally, also, there has been a persistence of purulent nasal discharge, for days or weeks, suggesting a chronic paranasal sinusitis; this has been particularly true of one of the animals which has a large polypoid growth in the posterior naso-pharynx.

Of considerable interest to us have been the findings with respect to the animals' apparent immunity succeeding colds. Rarely have they been given or have they caught colds in less than 3 months after having a previous infection, and in most cases the interval between infections has been longer. This has suggested the possibility that there is a period of insusceptibility or of immunity of 3 or 4 months' duration, in these animals, succeeding colds. As a result of these findings we no longer attempt transmission experiments during the 3 or 4 months that follow a cold, as a negative result would be of little significance. One important exception to the foregoing has been noted. In December, 1928, three of our animals acquired acute upper respiratory infections 3 or 4 weeks after having had colds. Clinically, however, these infections closely resembled the influenzal type which was then epidemic and from which our animal men were suffering. Although very slight, this evidence points to the assumption that influenza and the common cold are separate entities.

The Bacterial Flora of the Upper Respiratory Tracts of Chimpanzees.— Soon after acquiring the apes a study was commenced to determine the bacterial content of their noses and throats. Swab cultures were taken from the nose and posterior naso-pharynx and were plated upon fresh rabbit's blood agar. The percentage incidence of the bacteria present in health is shown in Table I. For comparison we have

included in the table our earlier finds, 1924–25, in the case of man (1); the cultural methods used were the same in both cases (Table I).

It will be noted that the bacteria of the throat are surprisingly similar in man and ape. The usual basic organisms, non-hemolytic streptococci and Gram-negative cocci are identical in incidence. *B. pfeifferi* and hemolytic streptococci are higher in the ape than in man. *B. coli* appears in the ape flora as a result of the habits of the animal. Other differences are of slight degree only.

In the nasal cultures, staphylococci which are usually the predominating organisms in man are the same in incidence in the ape.

TABLE I

Comparison of Percentage Incidence of Bacteria in Noses and Throats of Normal Chimpanzees and Humans

		Organisms												
		Gram-negative cocci	Streptococcus non-hemolytic	Bacillus "X"	*B. pfeifferi*	Diphtheroids	Large Gram-positive cocci	*Staphylococcus albus*	Streptococcus hemolytic	*Staphylococcus aureus*	Pneumococcus	*B. coli*	Strepto-bacillus	Total cultures
Nose	Ape	17	60	0.6	13	21	8	95	1.2	26	1.2	4	15	169
	Man	1	7	0.0	0	79	0	92	0.4	36	0.0	0	0	265
Throat	Ape	99	99	21.0	92	11	5	31	45.0	9	0.6	13	0	159
	Man	99	99	49.0	47	45	45	40	17.0	14	2.0	0	0	265

Diphtheroids, very characteristic of the human nasal flora, are rather lower in percentage incidence in the ape. Strepto-bacilli seem to be peculiar to the ape and outside of the accidentally present *B. coli* seem to constitute the only qualitative bacterial difference. The higher percentage in the noses of apes of certain organisms such as Gram-negative cocci, non-hemolytic streptococci, *B. pfeifferi*, etc., which are usually regularly present in the throats of both man and ape are probably due to anatomical differences.

Gram-negative, filter-passing anaerobes also have been cultivated from nasal washings obtained from apes. Careful study of the incidence of these organisms has not been attempted because of the difficulty of securing washings.

Summarizing: (1) Chimpanzees are susceptible to colds when exposed to such infections in humans. (2) The clinical manifestations noted when the animals catch cold closely resemble those seen in man. (3) The bacterial flora of the nose and throat of these animals is very similar to that of man.

From the foregoing it seems clear that chimpanzees are suitable animals for the experimental transmission of colds.

II. Transmission Experiments with Chimpanzees

Having assured ourselves of the suitability of chimpanzees for our purposes we proceeded with attempts to transmit colds from human sufferers to apes by means of filtered nasal washings.

Methods.—Animals to be used were placed in the strict quarantine described above and were held there for several days before being used. This was done for the purpose of testing the efficiency of the isolation and for the exclusion of possible latent respiratory infection.

At the conclusion of this period of preliminary observation, which lasted 5 days or longer, individuals suffering from suitable colds were sought out. The type of cold selected was one of not more than 24 hours' duration and of moderate severity. A special effort was made to exclude colds that did not conform strictly to the classical clinical types, such, for example, as the so-called "grippy" varieties in which fever of other constitutional symptoms were manifest.

Nasal washings were obtained from the subjects with colds by gently running slightly warmed stock buffered broth (pH, 7.6) into their nostrils (5 to 10 cc. per side) and thence out through the mouth; 10 to 20 cc. of the broth was next gargled and added to the nasal washing. The material thus collected was shaken vigorously together with glass beads to break up the clumps of mucus. It was then passed through a Berkefeld V candle. The unfiltered material was cultured aerobically upon blood plates. The filtrate was cultured aerobically and anaerobically upon blood agar plates and blood broth and in the Smith-Noguchi medium to determine its sterility and the presence of filter-passing anaerobes. Further, as a control measure, 0.25 cc. of filtrate was injected intracerebrally or intracisternally in rabbits to exclude the presence of herpes virus.

As soon as possible after the filtration of the nasal washings, 1 cc. of the filtrate was injected with careful aseptic precautions into each nostril of the quarantined chimpanzees. For each experiment it was customary for us to collect two nasal washings at intervals of a few hours and to make two intranasal inoculations in each experimental animal with the filtrates obtained from these. The time elapsing between the collection of each washing and its injection into the ape was usually less than 1 hour.

Results.—In the chimpanzee experiments performed during the last 2 years we have had 44 per cent of successful transmissions by means of filtrates. A summary of these appears later. In positive experiments, the first symptoms of the cold have appeared within 36 to 48 hours following inoculation. A typical successful transmission experiment is shown in Fig. 1. On this chart appear both the clinical and bacteriological findings. The time of inoculation, the incubation

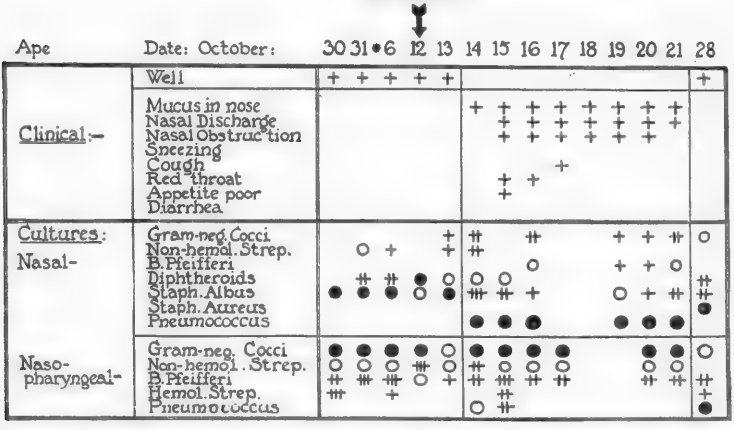

FIG. 1. Chart showing development of symptoms in an experimental cold after intranasal inoculation of a chimpanzee with filtered nasal washings from a human cold. The bacterial flora in nose and throat before, during, and after is shown graphically. Solid circles indicate predominating organism, blank circle the next most numerous, plus signs indicate the remainder.

period and the sequence of symptoms are shown graphically in the upper portion and are self-explanatory.

On the lower half of the chart are recorded the bacterial findings and these are of considerable interest. It will be noted that coincident with the initiation of cold symptoms there is a definite alteration in the flora. This is most striking in the nose cultures, where pneumococci (Type IV) have become the predominating organism, and *B. pfeifferi* has become conspicuous. In the throat cultures, pneumococci suddenly appear.

Alterations of this type, in which pneumococci have suddenly ap-

peared in the nose and throat cultures, or where *B. pfeifferi* and occasionally hemolytic streptococci have spread to the nose at the start of colds, have been present almost always in both the induced and the accidental infections in apes. In our studies of the flora in humans both in our previous, and the present work, we have not observed this phenomenon. The contrast between the slight qualitative changes in man and the striking alteration in the ape is shown in Table II.

TABLE II

Comparison of Percentage Incidence of Bacteria in Noses and Throats of Chimpanzees (a) during Normal Periods and (b) in the Course of Colds

		Organisms												
		Gram-negative cocci	Non-hemolytic streptococcus	Bacillus "X"	*B. pfeifferi*	Diphtheroids	Large Gram-positive cocci	*Staphylococcus albus*	Hemolytic streptococcus	*Staphylococcus aureus*	Pneumococcus	*B. coli*	Strepto-bacillus	Number of cultures
Nose	Normal	17	60	0.6	13	13	8	95	1.2	26	1.2	4	15	169
	Colds	38	33	0.5	59	9	2	87	5.0	11	49.0	10	25	172
Naso-pharynx	Normal	99	99	21.0	92	11	5	31	45.0	9	0.6	13	0	159
	Colds	100	99	17.0	94	9	8	18	36.0	2	14.0	21	0	104

Early in the course of the work control experiments were begun, intranasal inoculations of plain broth and of heated filtrate being used. These were soon given up as it was believed that filtered nasal washings from normal individuals who were free from respiratory infection would be more suitable for control purposes. In order to reduce the likelihood of including carriers of the active agent, as sources of normal nasal washings, the summer months were selected for this group of experiments, colds being at a minimum in this season.

The animals were quarantined in the usual manner and after the customary preliminary period of observation they were inoculated with the filtered nasal washings, the same procedure which had been used for the transmission experiments being carefully followed throughout. As a source of the normal nasal washings healthy individuals who had had no colds or sequelae for at least 3 or

4 months and who had had no known recent exposure to current colds were used. Eight experiments were done in all.

Following these inoculations no change in the health of the animals was observed. There was an entire absence of even small amounts of nasal mucous discharge and no constitutional manifestations whatever were observed. In marked contrast with the characteristic alterations in the bacterial flora of the noses and throats of animals suffering from experimental or spontaneous colds, no changes were noted in these control animals.

The filtered nasal washings, obtained from individuals with colds and used to transmit these infections to apes, contained Gram-negative filter-passing anaerobes in a high proportion of cases. This is as it should be, if, as we believe, these organisms constitute part of the basic flora of the nose and throat. The fact that these organisms were present in 75 per cent of the control (normal) filtrates, which caused no symptoms whatever in the animals inoculated, provides further evidence that they have very doubtful etiological significance in colds. It is true that their incidence in the filtrates has been higher (86 per cent) in the positive experiments than in the negative (55 per cent). However these figures were reversed in our previous studies (2), and, as will be pointed out below, the organisms were present in equal proportions (100 per cent) in both our negative and positive human transmission experiments. That certain specific types, occurring in this extremely heterogeneous group of organisms may be found to possess etiological significance is not impossible. So far we have no evidence in support of this assumption.

In the course of the present work, 36 ape experiments have been completed. These may be divided into two groups. First, 28 experiments concerned directly with the testing of the hypothesis that colds may be caused by filtrable agents, and second, 8 miscellaneous experiments.

In the first group washings from individuals with colds were used in 20 instances and normal washings in 8. Of the former, 4 animals were excluded for statistical purposes for reasons given below. Of the remaining 16 animals, 7 contracted colds, 1 atypical; this represents a 44 per cent incidence of successful transmissions. The four experiments referred to above were not included because the inoculations

were made in animals just purchased which had recently suffered from respiratory infections. This has been done in the light of our present recognition of the post-cold insusceptibility referred to above. All eight of the control experiments, as noted above, were completely negative.

In the miscellaneous group, one was an experiment done with unfiltered nasal washings. It was positive. Two were ape to ape experiments, in which filtered ape washings were used. They were both positive. In order to determine the viability of the active agent three experiments were done with filtrates which had been stored in the ice-box 2 to 7 days. These were all negative. The remaining two experiments were done with living cultures of the filter-passing anaerobes obtained from a filtrate which had been used in a successful transmission experiment, these were both negative.

Finally, it is of importance to call attention to the following. On several occasions apes were in the quarantine room with others which were being used for transmission experiments. Several times when the latter had acquired experimental colds, the uninoculated animals contracted the infection in 3 or 4 days, presumably as a result of contact.

III. Transmission Experiments in Man

Having to our satisfaction worked out the above described effective quarantine technique for carrying out transmission experiments with apes, we considered it desirable to apply the same methods to man. We felt further that if we should utilize this rigorous isolation technique with man we could meet the usual criticism directed against human transmission experiments, as previously performed, without such strict quarantine.

Methods.—Human volunteers were secured through the employment bureau of the Hospital. Young adults who stated that they had had no colds or other respiratory infections in recent months and who had never had sinus complications or pneumonia were chosen. Reasonable intelligence and familiarity with the English language were also stressed in the selection of the volunteers.

The subjects were given a bath and were put into hospital clothes which had been autoclaved. They were placed immediately in an isolation room. They were not permitted to leave this room before the completion of the experiment.

The room had been previously cleaned with 2 per cent lysol solution and then aired for 24 hours. Everything in the room, including linen, toilet articles, occupational therapy material, with which the subjects whiled away their time, reading matter, etc., was carefully sterilized. Food entering the room was sterilized wherever possible.

The volunteers were attended by graduate nurses who were thoroughly versed in the principles of aseptic surgical technique and who always wore cap, gown, mask and rubber gloves upon entering the isolation room, and who used every precaution to prevent the possibility of entrance of infection from outside. Further, the visits of both nurses and workers were reduced to a minimum.

Upon entering quarantine the subjects were given a thorough physical examination, and a study, by daily culture, was begun of the flora of their noses and throats. In addition their sputum was tested by the mouse injection method for the presence of pneumococci. This was done as the finding of fixed types of these organisms was considered to be a contraindication to the use of the individual for inoculation.

As in the case of the apes, these human subjects were held for 5 days or more for preliminary observation, this being done to test the efficacy of the isolation and to exclude entrance during possible incubation of an upper respiratory infection.

At the termination of this period of observation, if the volunteer proved satisfactory, suitable colds were selected, washings obtained and filtered, and intranasal inoculation carried out in the manner described above for the ape experiments. For injection of the filtrate the subject was placed in the dorsal position for 2 to 3 minutes and then was turned over on his face for 1 minute.

Results.—Altogether 11 men were used in succession for this form of experiment. Two of the experiments were not completed. One because the attendant nurse contracted a cold just at the end of the preliminary period of observation and the other because the subject was found to be a carrier of Type III pneumococcus.

Of the 9 completed experiments 4 were positive. This represents a 44 per cent incidence of successful transmissions, a percentage incidence which is practically identical with that obtained in the work with anthropoids. A brief description of these colds will be given below. Before proceeding to do this, attention should be called to certain aspects of this type of experiment which are worthy of emphasis.

It is very easy for an individual who is being used for a transmission experiment to believe that he has a mild cold although objective evidence is extremely slight or absent. Where, as in the beginning of our work, volunteers believed that we were trying to produce colds, they were self-convinced occasionally that they were suffering from a

mild infection. This was much easier of belief as the filtrate in practically all the cases, negative and positive, causes some slight stuffiness of the nose, a little sneezing and occasionally slight headache.

Very early in the work we recognized this willingness of our subjects to oblige us and began taking measure to avoid this source of error. By various ruses, as nasal injections of sterile broth, collection of nasal washings for culture and equivocal statements, we were enabled to keep the subjects in ignorance. In certain instances, where the intelligence of the subjects was commensurate, we sought their direct cooperation.

A further help in ruling out error was dependence upon concrete objective findings. Such signs and symptoms as injection of the conjunctivae, profuse nasal discharge, muco-purulent post-nasal discharge, frank inflammation of the pharynx with lymphatic hyperplasia, continuous cough, etc., were considered indispensable for the interpretation of a result as positive.

Case H 4 is worthy of note as an example in this connection.

It was apparent very early that this individual was more or less unreliable and from the start it was possible to keep him in the dark regarding our procedure. He had inconspicuous symptoms after his test injection of sterile broth and no more striking results from the cold filtrate, until an assistant, on the second day after injection, inadvertently referred to his failure to contract a cold. That evening and night the subject reported severe symptomatology, including sneezing, cough, sore throat and stuffiness of the nose. The next morning he was told that he had been misinformed in regard to the nature of the filtrate and his symptoms subsided within the hour. It is important to note that there was an entire absence of objective pathological changes.

The 4 experimental colds that occurred in this group of 9 attempted transmissions, appeared in Cases 1, 3, 5 and 9 of the series. The incubation period in all cases was 24 hours or less. The first and third of these are shown in graphic fashion in Figs. 2 and 3.

Fig. 2 shows the 5 day period of preliminary observation, the time of inoculation and the appearance of symptoms after a little over 12 hours. The cold was a rather severe one, was much better on the fifth and sixth days (as shown on the chart) and then became considerably worse 2 days after release from quarantine. This finding will be referred to below. The bacteriological observations are shown on the lower half of the figure. At the onset and during the course of the cold

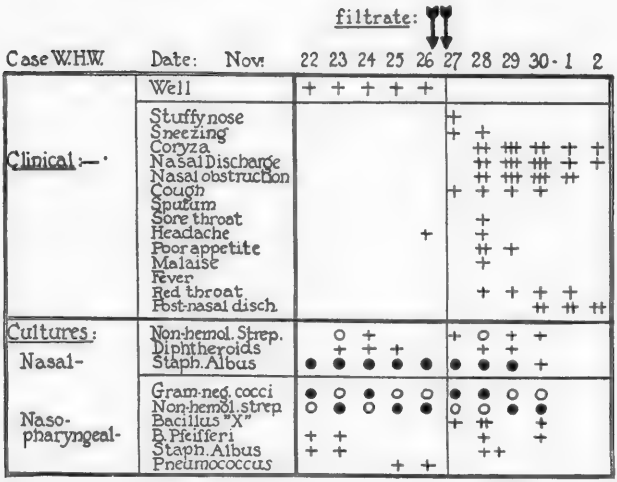

FIG. 2. Experimental cold in man, showing clinical and bacteriological development. Symbols as in Fig. 1.

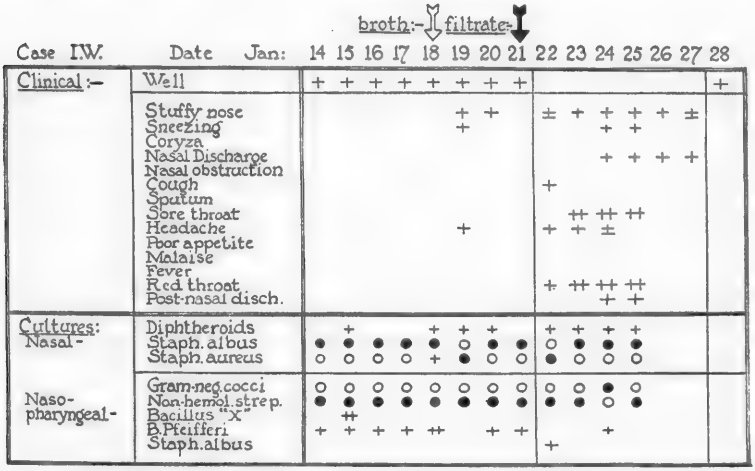

FIG. 3. Experimental cold in man, showing results of preliminary sterile broth inoculation. Symbols as in Fig. 1.

there is no essential change in the characteristic flora of the nose and throat. The presence of pneumococci (IV) in the period of preliminary observation is probably without significance as we have observed this both in our normal studies and in the present experiments where results have been negative.

Fig. 3 shows a rather different type of cold. The symptoms of rhinitis and coryza were mild while the pharyngeal ones were quite conspicuous. The following is of much interest in this particular instance. While one of us was collecting the nasal washing for this case the patient coughed vigorously directly in worker's face. 2 days later, a few hours after the onset of the experimental cold this worker came down with an infection which was very similar, especially with respect to the comparatively negligible nasal symptoms. This figure shows also a typical result when sterile broth is injected as a preliminary step. Slight stuffiness of the nose and headache and a little sneezing appeared. These had completely subsided by the third day and the subject was quite free of symptoms when he received the cold filtrate. It will be noted again that there are no changes of importance in the bacterial flora.

Of the remaining two colds, one was a mild one in which sore throat, laryngitis and cough with moderate amounts of sputum, were conspicuous. The other was a simple uncomplicated mild cold. In these colds also no significant alterations in the bacterial flora, incident to the infections, were noted.

Of much interest to us has been the sequence of events in two of the three colds which we have been able to follow after release from isolation. In both these there was a definite exacerbation of the infection a day or two after leaving. This has been considered to be the probable result of protection from exposure to transient potential secondary invaders provided by the quarantine. The individual upon leaving again is accessible to such potential pathogens which become active upon the substrate of the experimental cold. In the light of the above, the volunteer who acquired the last experimental cold and who was the third of those whom we were able to follow was advised to stay away from crowds for a few days after being discharged. It is interesting but not necessarily significant that he had no recrudescence of his symptoms.

As noted above, filter-passing anaerobes were present in all cold filtrates used in these experiments irrespective of their outcome. Further, their type distribution was approximately the same in filtrates resulting in both positive and negative transmission experiments. This observation does not preclude the possibility that these organisms

play a part in the causation of colds, but it does constitute another link in the chain of evidence against this probability. Further studies directed toward more precise determination of the possible relationship of these organisms to colds are being pursued.*

DISCUSSION

Three points seem to be worthy of especial emphasis.

1. Chimpanzees would seem to be, from the foregoing, unusually satisfactory animals for use in the study of infection of the human upper respiratory tract. When they contract these infections, the clinical picture they present is strikingly similar to that observed in man. This taken together with the fact that their bacteriological flora is essentially like that of man and that they are closely related biologically to man suggests that their pathological and immunological response to bacteria pathogenic for the human upper respiratory tract may be sufficiently similar to make them the ideal experimental animal for this type of study. The tractability and cooperativeness of the chimpanzees makes them still more desirable. When well cared for they seem to keep in good health for long periods of time.

2. Colds may be transmitted to man and chimpanzee by intranasal inoculation of filtered nasal washings. These filtrates usually can be shown to contain anaerobic filter-passers of the type first described by Olitsky and Gates. However, our evidence so far points to the probably non-pathogenicity of these organisms for man. That specific types in this group may be shown later to play a part in causing colds has been pointed out above. If, however, as seems most probable at present, proof of this is not forthcoming, it follows that the active agent present in these filtrates, by means of which we have been able to transmit colds, is a true submicroscopic virus.

3. The sudden and plentiful appearance of pneumococci in the noses and throats of the chimpanzees in the course of colds has been most striking. The nature of this response is difficult of interpretation. There is little doubt that these organisms have been present

*Since this article was written the number of experimental colds transmitted by filtered nasal washings from which filter-passing Gram-negative anaerobes have been absent has increased so that it is now certain that colds can be transmitted by bacteria-free filtrates from the sources described.

before the infection, spontaneous or experimental, of the animal. They have been noted often in small numbers at various intervals in the throats of the animals and might well be shown to be regularly present by the mouse injection method. We have felt that their prominence may well be the result of multiplication and spread upon a substrate of primary injury due to the filtrable agent or that there may be some sort of activation of these potential pathogens by this agent. A like explanation may be offered for the spread to the nose of *B. pfeifferi* and hemolytic streptococci.

CONCLUSIONS

1. Chimpanzees are highly suitable animals for the experimental study of human upper respiratory infections.

2. Human colds have been successfully transmitted to apes and human volunteers in 44 per cent of instances tried by means of filtered nasal washings obtained from colds.

3. Certain types of infectious colds are caused by a filtrable agent.

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STUDIES IN THE SEROLOGY OF SYPHILIS

I. The Mechanism of the Flocculation Reactions

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In alcoholic solution, the normal tissue lipoids used as "antigen" in the serum-diagnosis of syphilis are probably molecularly dispersed. Upon dilution with water, however, there is a marked aggregation of the lipoid molecules. The originally transparent solution becomes opalescent, gives a Tyndall phenomenon, and by darkfield examination, innumerable highly refractile lipoid particles in active Brownian motion can be seen. Their average size, the number visible, and therefore the degree of opalescence, depend upon the method of admixture of the two liquids (Sachs and Rondoni, 1909).

If, to a diluted antigen in which all these microscopically visible particles are discrete, one adds syphilitic serum, there is, as Jacobsthal (1911) observed, a rapid clumping of the apparently unchanged lipoid micellae into coherent aggregates. Depending upon the concentration of the reagents, the temperature, time of incubation, etc., these aggregates may or may not exceed the limits of colloidal stability to form the optically visible "precipitate" first noted by Michaelis (1907). As he predicted, this precipitation phenomenon has found wide-spread diagnostic application. The Sachs-Georgi and Kahn precipitation tests, the Vernes flocculation reaction, the Murata test, the Sigma reaction, the Meinicke-Trübungs, Meinicke-Klärungs, and Müller-Ballungs reactions, and the Hinton agglutination reaction are all basically the same. The lipoid antigen, modified by the addition of tolu balsam, cholesterol, glycerol, NaCl, etc., forms a more or less stable "solution" (suspension) in normal serum; but in syphilitic serum there is a visible aggregation.

* Aided by a grant from the Committee on Research in Syphilis, Inc.

The causes of this aggregation and the optimal conditions for its production are the subjects of the present paper.

I. Surface Properties of the Lipoid Antigen

Colloids in general are divided more or less arbitrarily into two heterogeneous groups, between which there is no sharp distinction, but which, at the extremes, are quite characteristic (Svedberg, 1924): hydrophobic, with no affinity to water, and therefore readily flocculated by electrolytes (gold sol; Fe_2O_3 sol), and hydrophilic (proteins; glycogen).

The lipoid antigen in alcoholic solution is probably molecularly dispersed. Diluted with water, however, the molecules aggregate to form a colloidal suspension, the properties of which are intermediate between those of the hydrophobic and hydrophilic classes. The colloidally dispersed lipoid is amphoteric, flocculating at its isoelectric point, pH 1.9 (Fig. 3), but relatively stable at more alkaline reactions. Thus, at pH 6.0 (Figs. 1 and 2) it requires about 1 N concentration of univalent cations to cause aggregation of the dilute sol (0.04 per cent), and, as usually found with negatively charged particles, about 1/40 as much of bivalent cations ($BaCl_2$). The critical potential, the minimum compatible with stability, is 2 to 5 millivolts, much lower than that of most hydrophobic colloids, and strongly suggesting some other factor making for stability, analogous to hydrophilic colloids.

Protocol 1

The procedure followed in the preparation of a concentrated antigen was in essential details that used by Kahn (1925). 250 gm. of dehydrated powdered beef heart were shaken for 10 minutes with 1000 cc. ether, the filtrate discarded and the process repeated twice again with the residue. The dry powder remaining was weighed, and extracted for 3 days with 95 per cent ethyl alcohol (5 cc. per gram). Cholesterin is added to the pale yellow filtrate to any desired concentration.

5 cc. of antigen containing 1.5 per cent of beef heart lipoid and 0.2 per cent of added cholesterin were shaken with 5 cc. of NaCl N/7, and the suspension centrifuged. The sediment is shaken up in 10 cc. H_2O, forming a suspension containing approximately 0.7 per cent lipoid, varying quantities of which are dropped into electrolytes of various concentration. The pH is kept within an approximate range by phosphate buffers (final concentration M/300), the exact value being determined potentiometrically. The total volume is brought up to 4 cc. with H_2O, the tubes shaken, and flocculation read after 24 hours at room temperature. The data are summarized in Figs. 1 and 2.

One other point should be mentioned here, which will be referred to again in another connection. The concentration of electrolytes necessary to produce flocculation (coagulation value) is not a fixed quantity, an intrinsic property of the given colloid, but varies markedly with the concentration of the sol. As seen in Table I and Fig. 2, the more particles per unit volume, the less stable is the suspension. A smaller concentration of electrolyte suffices to produce aggregation; and the

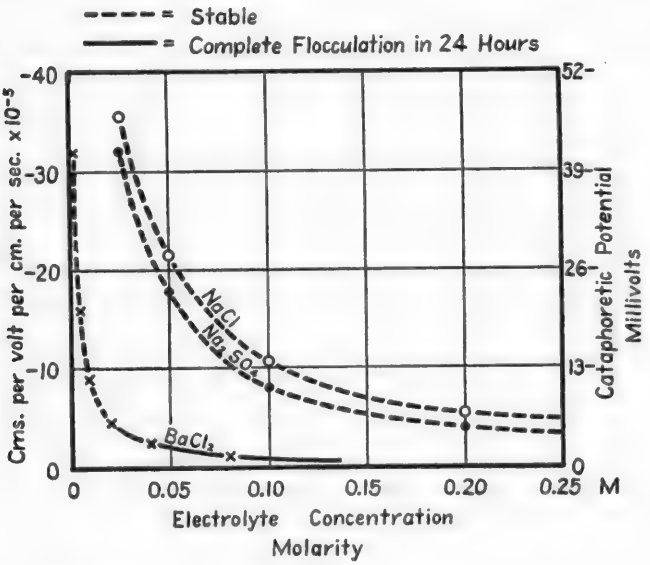

FIG. 1. Effect of electrolytes upon electrokinetic potential and stability of lipoid sol (0.04 per cent) at pH 6.0.

surface charge necessary to keep the particles dispersed and thus ensure stability becomes progressively higher. The theoretical considerations involved are discussed by Freundlich (1922).

II. The Effect of Normal Serum upon the Lipoid Antigen

Serum proteins in solution are, of course, highly hydrophilic. When adsorbed at the surface of a heterogeneous phase, however, they may act in either of two diametrically opposed senses, depending upon the

nature of the absorbent. They may retain their hydrophilic properties and form a protective film around a hydrophobic particle, preventing its flocculation by electrolytes; or they may become denatured when adsorbed, lose their hydrophilic properties, and become instead water-insoluble, hydrophobic; they then sensitize the adsorbing particle, making it even more susceptible to flocculation by electrolytes.

The nature of this denaturation is not clearly known; full discussions are to be found elsewhere (Freundlich, 1922, 1924). The point of interest is that serum globulin, when adsorbed by a colloidally dispersed heterogeneous phase, may form either a protective or sensitizing

TABLE I

Effect of Increasing Concentration of Lipoid Sol (Antigen) upon Its Stability and Coagulation Value (Fig. 1)

NaCl, normality	0.1	0.2	0.4	0.8	1.6
Concentration of lipoid in sol:					
0.002 per cent	0	0	0	0	2
0.008 per cent	0	0	0	1	2
0.032 per cent	0	0	0	3	4
0.128 per cent	1	1	3	4	4
0.512 per cent	3	3	4	4	4

Figures in body of table indicate agglutination after 24 hours.
4—complete, with clear fluid.
3, 2, 1—degrees of aggregation.
0—no optically visible aggregates.

film, depending upon the nature of the adsorbent, the determining factors being unknown.

When the lipoid antigen is added to normal serum, one can demonstrate a remarkably avid adsorption of the serum protein by the particles of the colloidally dispersed lipoid.

Protocol 2

5 cc. of antigen were shaken with 5 cc. of NaCl N/7, the suspension centrifuged to remove the alcohol, and resuspended to 10 cc. in H_2O. Series of tubes were set up similar to those outlined in Tables II and III, differing only in the concentration of normal, *i.e.*, Wassermann and Kahn negative serum. Cataphoretic velocities were measured in the simple Michaelis (1926) chamber, care being taken to make

readings one-fifth of the distance from the top and bottom of the chamber to avoid endosmotic currents, and in both directions to counteract drifts due to gravity. The values given for the velocity in the tables and in the figures were obtained by measuring the time required for the particle to pass between two points on an ocular-micrometer scale. The absolute values of this velocity of the electrokinetic potential it implies (see note to Tables II and III) have only a qualitative significance, the important factor being the isoelectric point, the hydrogen ion concentration at which the charge on the particle changes sign (equal ionization as acid and base), and at which there is therefore no movement in an electrical field.

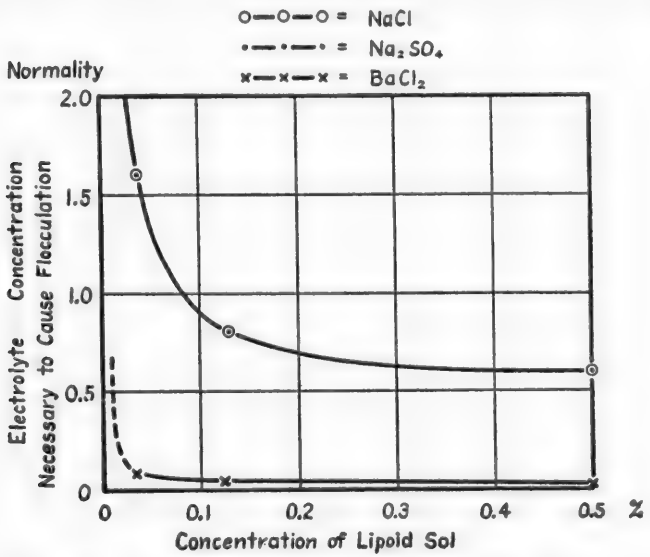

FIG. 2. Effect of concentration of lipoid upon its stability (pH 7.4).

Hydrogen ion concentrations were determined with the quinhydrone electrode, the error being ±0.05 pH.

The solid lines in all the figures indicate zones of flocculation, uniformly corresponding to zones of minimal potential; + signs indicate a particle positively charged towards the water, migrating to the cathode in an electrical field, while − signs imply a negative electrokinetic potential.

A. Isoelectric Point.—As little as 1:4000 serum (*i.e.*, 1:50,000 protein) suffices to alter the surface properties of the lipoid particles significantly. The zone of optimal flocculation, coinciding with the

cataphoretic isoelectric point, shifts from pH 1.9±, that of the lipoid antigen particles, towards a more alkaline reaction, the degree of shift depending upon the concentration of serum protein. It is significant that the maximum change is to pH 4.9, midway between the isoelectric points of serum albumen and serum globulin; the particle is then completely covered with protein, and has the same surface properties. With less serum, however, the amount of protein adsorbed does not suffice to cover the cell; the charge and the isoelec-

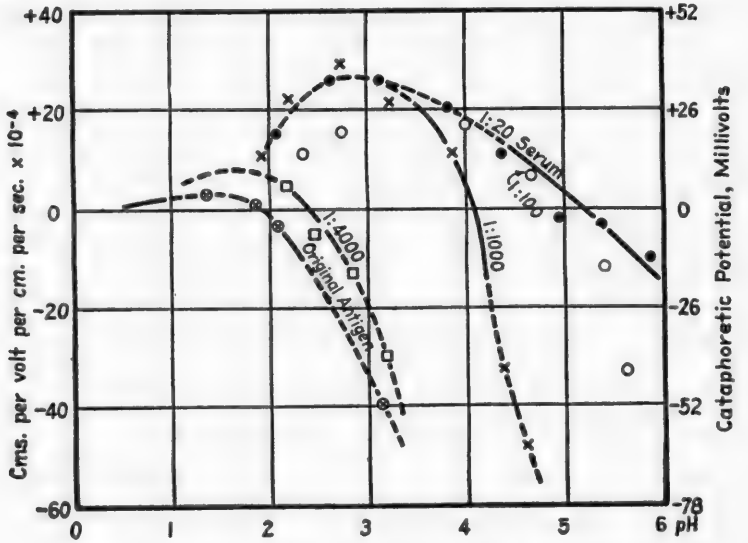

FIG. 3. Effect of normal serum upon cataphoresis of the antigen particles.

tric point, determined as they are by a mosaic of protein and lipoid, therefore have some intermediate value, the exact value of which depends upon the proportions of the two types of surface. This is more clearly shown in Fig. 4.

In addition to affecting the isoelectric point, this adsorbed normal serum protein changes the surface charge (and cataphoretic velocity) of the antigen at all reactions, corresponding to a change from a surface of lecithin to one of serum protein, the degree of shift again depending upon the extent of the adsorbed film.

TABLE II

Effect of pH upon Surface Properties of the Lipoid Antigen in Absence of Serum (Fig. 3, Curve ⊗—⊗)

HCl, N/1, cc.	3.8	1.0	0.25	0.062	0.05				
" N/100, cc.						1.6	0.4	0.1	0.025
H_2O, cc.	0	2.8	3.55	3.75	3.75	2.2	3.4	3.7	3.8
Antigen suspension, cc.	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
pH	<1	<1	1.35	1.86	2.0	2.51	3.14	3.8	4.56
Cataphoretic velocity cm. per (volt per cm.) per sec. $\times 10^{-5}$	<1	+1	+3.6	+1.4	−3	−16	−40	>−80	>−80
Surface charge* millivolts	<+1	+1.3	+4.7	+1.8	−4	−20	−52	>−100	>−100
Agglutination in 2 hours	±	0	+	++++	+++	0	0	0	0

TABLE III

Effect of pH upon Surface Properties of the Lipoid Antigen in 1:1000 Normal Serum (Fig. 3, x − x Curve)

HCl, N 1/10, cc.	1.28	0.64								
HCl, N 1/100, cc.			3.2	0.8	0.4	0.2	0.1			
HCl N 1/1000, cc.								0.8	0.4	0.2
H_2O	3.2	3.86	1.3	3.7	4.1	4.3	4.4	3.7	4.1	4.3
Antigen suspension + 1:100 serum	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
pH	1.64	1.93	2.21	2.88	3.23	3.88	4.38	4.6	5.14	5.4
Cataphoretic velocity cm. per volt per cm. sec. $\times 10^{-5}$	+2.5	+10	+22.4	+29.1	+21.3	+10.3	−32	−48	>−80	>−80
Electrokinetic potential,* millivolts	+3	+13	+27	+38	+28	+11.4	−39	−62.4	>−100	>−100
Macroscopic agglutination in 2 hours	0	0	0	0	0	±	+++	±	0	0

\+ = Positive charge; migration to cathode.

− = Negative charge; migration to anode.

* Electrokinetic potential (electrostatic units) $= \frac{4\pi\eta}{KX}$ u

$$= \frac{4\pi \times \text{coefficient of viscosity}}{\text{dielectric constant} \times \text{potential gradient (E.S.U.)}} \times \text{velocity}$$

$$\text{Potential (volts)} = 300\left[\frac{12.57 \times 0.0093\ (23°\text{C.})}{80}\right] \times \text{velocity per volt per cm. per sec.} \times 300$$

$$= 13 \times \text{velocity per volt per cm. per sec.}$$

Protocol 3

To each of a series of tubes were added 0.2 cc. of phosphate buffer (M/15) at pH 7.4, varying quantities of serum, and H_2O to 4 cc. A similar control series was prepared, containing varying quantities of NaCl 0.85 per cent instead of serum as in the first series. A washed suspension of antigen was added to each tube and cataphoretic velocity determined. The experimental data are summarized in Fig. 5.

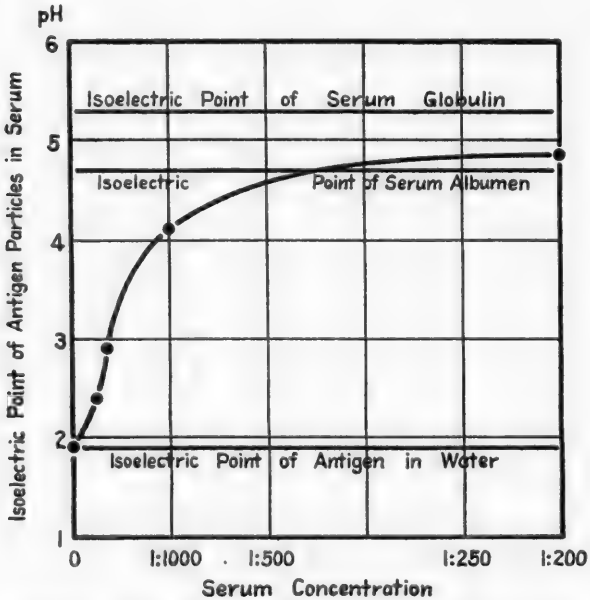

FIG. 4. Isoelectric point of antigen particles in serum is determined by adsorbed serum protein.

B. Flocculation.—Despite the fact that, as just shown, serum protein is strongly adsorbed by the lipoid antigen, there is no significant change in its stability, as determined by its tendency to flocculate. True, the isoelectric point, and therefore the range of optimum flocculation, shift towards that of the adsorbed protein: but away from this reaction the suspension is even more stable than before the addition of serum. The adsorbed protein remains hydrophilic, and acts as a film of pro-

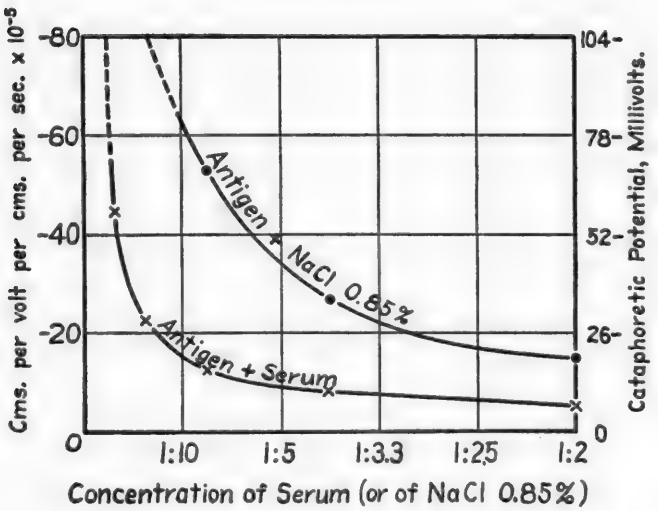

FIG. 5. Effect of normal serum upon surface properties of antigen at pH 7.4.

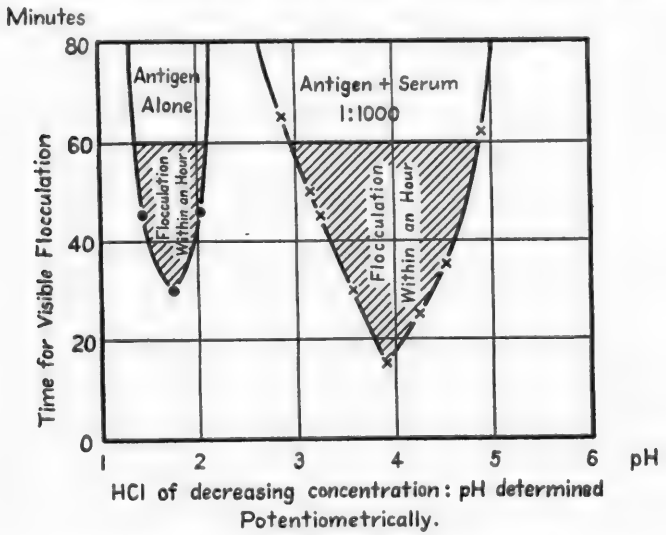

FIG. 6. Effect of normal serum upon flocculating properties of antigen.

tective colloid, preventing even the normal flocculation of antigen at its own isoelectric point (Fig. 6): while at serum reaction (pH 7.4) just as much electrolyte is required to cause flocculation in the presence of serum as in its complete absence.

Possibly, at high electrolyte concentration (*e.g.*, > M/2) the adsorption of normal serum protein is prevented, allowing the flocculation of the uncovered lipoid particle by electrolytes. At any rate, the critical potential and the coagulation value, and therefore stability, are not affected by normal serum.

To summarize, normal serum protein is strongly adsorbed by the lipoid antigen,* forming a protective film of hydrophilic protein around the constituent particles, and adding to their stability away from their isoelectric point.

III. Properties of the Lipoid in Syphilitic Serum

When the antigen is placed in syphilitic serum, there is, of course, the same non-specific adsorption of normal serum protein. In addition, however, it combines with a specific component, so-called "reagin," with a striking change in its surface properties.

A. Composition of the Lipoid-Reagin Precipitate.—

Like true antibodies, this "reagin" is always associated with the globulin fraction of serum protein (Kapsenberg, 1924) (Sahlmann, 1922) (Gloor and Klinger, 1920). Such evidence as there is to the contrary (Felke, 1921; Skrop, 1923) has been vigorously discredited by Stern (1923). *A priori*, then, the precipitate should consist of the antigen plus the reagin-globulin with which it has combined. On this point, however, there is conflicting evidence.

* This adsorption of normal serum protein at all reactions is in itself the strongest evidence against the theory (Epstein and Paul, 1922) that syphilitic reagin is a positively charged colloid, which combines with the negatively charged lipoid particle, with mutual discharge and precipitation. There is no experimental evidence for this theory (Bauer and Nyiri, 1921), (Stern, 1923, 1924); moreover, it fails to explain why flocculation is not obtained when syphilitic serum is added to any negatively charged suspension. The fact that normal protein is adsorbed by lipoid even when both have the same charge (both negative at serum reaction) shows that one can not predicate opposite charges as the cause for the combination of lipoid with reagin, and their subsequent flocculation.

Jacobsthal (1911) considered the aggregates to the lipoidal, as did Niederhoff (1921), and Epstein and Paul (1921, 1922). Meinicke (1919), on the other hand, misled by his two-phase reaction, considered the precipitate to be entirely proteid, disregarding the fact that microscopically one can see the aggregation of the lipoid particles. Following Georgi (1919), who showed that the precipitate is not completely soluble in alcohol and ether, the exact analyses of Klostermann and Weisbach (1921) indicate that about 20 per cent of the precipitate can not be redissolved in alcohol and ether, and that of the insoluble residue at least one half (10 per cent of the total) is serum globulin; while Scheer (1921) considers the globulin to constitute 36 to 60 per cent of the total precipitate.

The following qualitative experiment shows clearly that the precipitate does consist chiefly (80 to 90 per cent) of the antigen lipoid, plus some specific component of syphilitic serum with which it has combined, a substance known to be associated with the globulin fraction of serum, giving the routine tests for protein and which can therefore be called reagin-globulin.

Protocol 4

Preparation of Large Quantities of Antigen-Reagin Precipitate.—5 cc. of the alcoholic extract containing 0.6 per cent cholesterin were mixed with 5 cc. of NaCl N/7 and the white suspension thus obtained shaken for a few minutes with 100 cc. of strongly positive syphilitic serum. After ½ hour at 56°C. and 24 hours at ice box temperature the suspension was diluted several times with saline in order to facilitate sedimentation, and centrifuged at 2500 r.p.m. for 30 minutes. The sediment was washed twice in 250 cc. NaCl N/7, once in H_2O and finally centrifuged in a graduated tube until its volume remained constant.

Qualitative Analysis of the Precipitate.—A known volume of sediment was extracted with 15 cc. of alcohol, then with 15 cc. of ether, and finally again with 15 cc. of alcohol. The residual precipitate was centrifuged in a hematocrit tube until its volume remained constant. As seen in Table IV, from 10 to 20 per cent by volume of the antigen-reagin precipitate is insoluble in alcohol and ether.

The combined alcohol and ether extractions, representing 80 to 90 per cent of the total precipitate, when evaporated to dryness and redissolved in alcohol, form as efficient an antigen as the original cholesterinized extract; the antigenic lipoids are carried down unchanged in the precipitate. The 10 to 20 per cent residue is insoluble in water and saline; but since it contains N, and give the routine tests for protein (biuret, Millon, xanthoproteic), it must consist in part, if not wholly, of denatured serum protein.

A quantitative gravimetric analysis of the precipitate has no real

significance. The proportion of alcohol-soluble lipoids to serum globulin will depend upon the reagin-titre of the serum used and the relative amounts of serum and antigen; moreover, such large percentages of protein as found by Scheer represent non-specific normal protein, incompletely removed by washing.

Protocol 5

8 cc. of saline were shaken with 8 cc. of cholesterinized antigen, the resulting suspension centrifuged, resuspended to 15 cc. and added with shaking to 100 cc. of strongly positive syphilitic serum. After 24 hours in the ice box the mixture was diluted with 300 cc. NaCl, N/7 (pH 7.4) and centrifuged. The sediment,

TABLE IV

Composition of Antigen-Reagin Precipitate

Antigen-reagin precipitate	Residue after extraction with alcohol and ether	Alcohol-ether soluble (volumetric)
cc.	*cc.*	*per cent*
0.2	0.038	81
0.5	0.05	90
2.0	0.28	86

taken up in 50 cc. NaCl, is Suspension 1, containing at most 1:50 parts of serum. After 2 hours at room temperature, the suspension was again centrifuged, and again made up to 50 cc. (Suspension 2, $<1:2500$ serum). The final suspension obtained after 4 such washings represents at least $1:10^7$ serum dilution, verified by the N content of the supernatant fluid.

The isoelectric point of each suspension was determined catophoretically and by the zone of optimal flocculation. The experimental data are summarized in Fig. 7, Table V corresponding to Curve 4 of the figure.

B. Effect of Washing.—

Normal serum alters the surface properties of the lipoid particles by virtue of the protein adsorbed. The following experiment shows that in the lipoid-reagin precipitate also, the protein detected chemically is present as a film around the lipoid particles, but more or less irreversibly adsorbed, and thus similar to antibody protein bound by a specific antigen.

Clearly, the protein taken up by the antigen from syphilitic serum is present as an incomplete film around the individual lipoid particles, not removed by washing. The isoelectric range of Suspension 4,

washed so thoroughly that it contains $<10^{-7}$ parts of free serum, is pH 3.4±, the same as antigen suspended in 1:2000 serum, instead of the normal value of pH 1.9. Once the normal serum has been removed (1 to 2 washings), repeated further washing makes no more change in the surface properties than is indicated by the shaded zone of Fig. 7. Assuming an approximately equal ionization of the lipoid and protein surfaces per unit area, the shift in isoelectric point to pH 3.4 implies a lipoid particle roughly one-half covered with protein.

TABLE V

Effect of pH upon Surface Properties of the Lipoid-Reagin Precipitate, Washed until Supernatant Is Practically N Free (Fig. 7, Curve □—□)

HCl, N/10, cc.	1	0.25					
" N/100, cc.			1.25	0.62	0.31	0.16	0.08
H_2O, cc.	2.8	3.55	2.55	3.2	3.5	3.65	3.70
Precipitate suspension, cc.	0.2	0.2	0.2	0.2	0.2	0.2	0.2
pH	1.64	2.2	2.52	2.81	3.13	3.56	4.22
Cataphoretic velocity cm. per volt per cm. per sec. $\times 10^{-5}$	+1.3	+10	16	+12	+10.8	−13.3	−40
Electrokinetic potential,* millivolts	+1.7	+13	+20.8	+15.6	+14	−17.3	−52.4
Agglutination in 2 hours	+	0	0	0	+++	++++	0

− = Negative charge; migration to anode.
+ = Positive charge; migration to cathode.
* See note at bottom of Table III.

Antigen therefore combines irreversibly with a specific protein of syphilitic serum. It is significant that Otto and Winkler (1922) arrived at the same conclusion by an entirely different line of investigation. They found that the washed lipoid-reagin precipitate sensitized 80 per cent of thirty-two guinea pigs to the subsequent injection of human serum, proving the presence of serum protein in the precipitate; while of the controls, nineteen guinea pigs injected with the lipoids washed after immersion in negative serum, only 20 per cent showed slight sensitivity to human serum, subsequently injected, and none died of anaphylactic shock.*

* Similarly, agglutinated bacteria (Braun, 1909) or red cells (Altmann, 1912) sensitize an animal to the subsequent injection of serum of the same species as the antiserum.

There is therefore a striking analogy to the so-called specific immune reactions. Bacteria, red cells, and lipoid antigen all adsorb normal, as well as antibody protein: but while the normal serum protein, remaining hydrophilic, is readily removed by washing, the antibody globulin is firmly bound, and has been shown (chemically, immunologically, and by surface properties) to form a more or less irreversible denatured film around the antigen (Eagle, 1929, 2, 3; 1930).

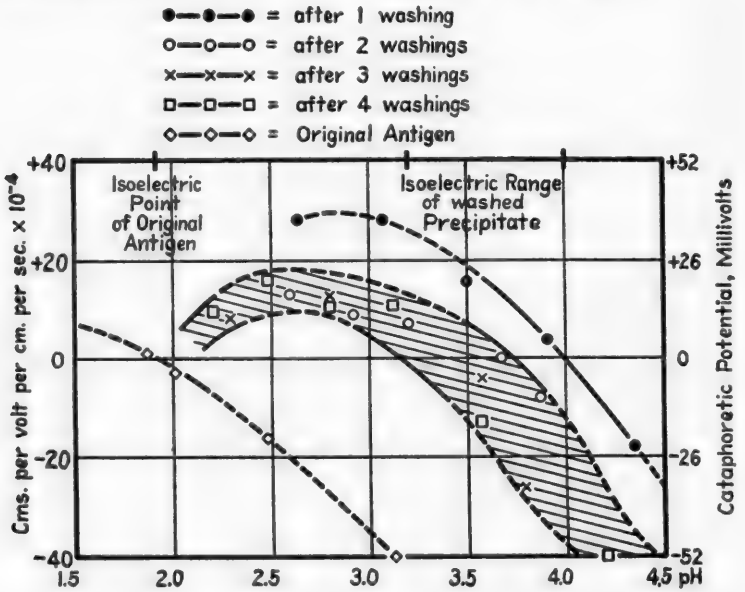

FIG. 7. Effect of washing upon the surface properties of the lipoid-reagin precipitate.

It has already been shown (Eagle, 1930) that the flocculation of bacteria or of red cells by the antiserum is due to this film of antibody protein, denatured by its combination with the antigen, and therefore sensitizing the antigen-antibody complex to flocculation by electrolytes. The obvious implication is that the flocculation of the lipoid syphilitic serum is an exactly similar process, an hypothesis which is confirmed in the following sections.

C. Flocculating Properties of the Lipoid-Reagin Complex.—
Normal serum protein affected only the isoelectric point and the cataphoretic potential of the antigen, without changing its critical potential. The adsorbed film retained its hydrophilic properties, and the suspension therefore remained stable up to about 1 M NaCl or 0.02 M $BaCl_2$, the exact coagulation value depending upon the concentration of lipoid.

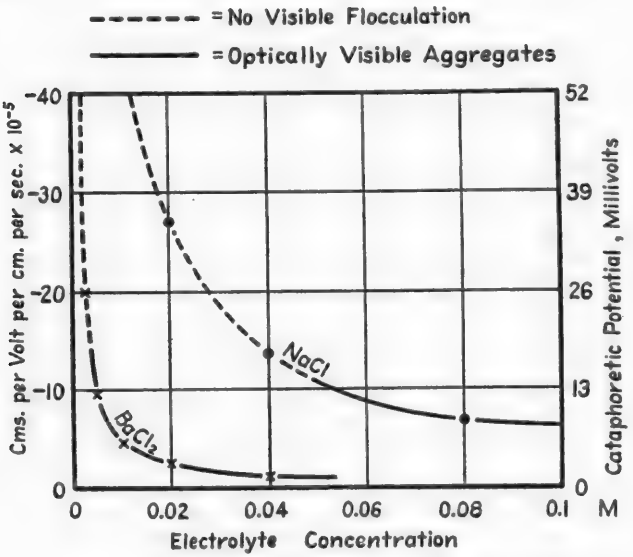

FIG. 8. Coagulation value of lipoid-reagin precipitate.

In marked contrast, the lipoid-reagin complex is flocculated at any hydrogen ion concentration by traces of electrolyte. The reagin with which the antigen particles combine not only changes their isoelectric point and lowers their cataphoretic potential exactly as does normal serum protein, but in addition, raises their critical potential from 1 to 5 millivolts to 10 to 15 millivolts (Figs. 8 and 9), an increase in cohesive tendency of approximately fivefold.

It is therefore more than a coincidence that such dissimilar particles as bacteria, red cells, protein micellae, and beef heart lecithin should

have exactly the same isoelectric point, cataphoretic potential, and in particular, the same critical potential, after sensitization with the homologous antiserum (antibacterial, hemolytic, precipitating and syphilitic respectively). In all these antigen-antibody complexes, the surface of the originally dissimilar antigen particles has been covered with an identical film of specific globulin, in some way altered by its combination so as to lose its affinity to the aqueous phase.

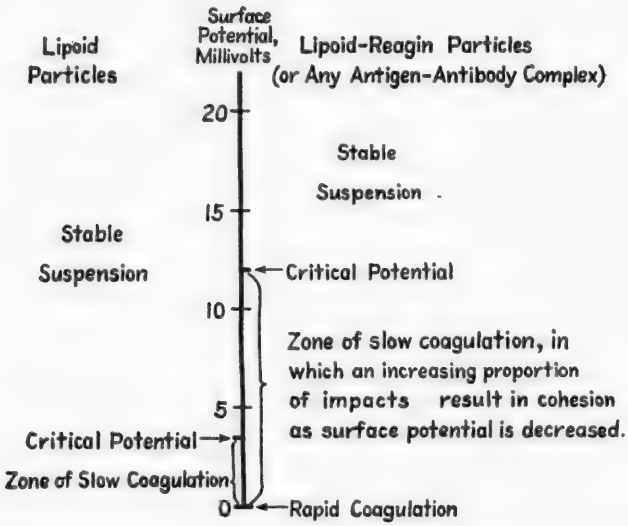

FIG. 9. Contrast between surface properties of antigen particles before and after combination with reagin.

At serum reaction, it ionizes as Na^+ globulinate$^-$, negatively charged because of the greater mobility of the inorganic ion. In the absence of electrolytes, this mutually repellent surface charge suffices to prevent cohesion of the particles as they approach during Brownian movement: the suspension is stable. Upon the addition of electrolytes the charge is depressed (Fig. 8). So soon as it falls below the critical value for denatured globulin (10 to 15 millivolts), the globulin-coated particles can approach within their radius of attraction, and aggregates form which sediment. The discharging ion is the one opposite in

charge to the particle, the cation; and salts with bivalent cations, which are 20 to 40 times as effective in discharging, are correspondingly more active in causing, flocculation. Thus (Fig. 8), the coagulation value for NaCl (or Na_2SO_4) is 1/20 to 1/15 M, for BaCl M/400 to M/200; quantities to be contrasted with the corresponding values for the original antigen suspension, before it acquired its sensitizing cohesive globulin film.

At first sight, it seems paradoxical that normal serum protein, when adsorbed, forms a protective hydrophilic film, while the reagin-protein serves to decrease the stability of the lipoid with which it has combined, by forming a sensitizing film of denatured hydrophobic protein. There are, however, many examples of serum protein serving in both capacities. Aside from the specific reactions already cited there is the action of protein-containing spinal fluid upon a colloidal solution of mastic or gold: in large quantities, the fluid may protect the sol against flocculation (hydrophilic film of protein), in smaller quantities, sensitize; there is the prozone in specific agglutination; the sensitizing action of globulin on Fe_2O_3 sols, and its protective action on dyes, etc. Moreover, the forces making for the loose reversible adsorption of normal serum protein are quite different from the specific affinity between the lipoid and the reagin globulin, as shown by the irreversibility of the latter combination, a difference further illustrated in the following section.

D. Complement-Fixing Properties of the Lipoid-Reagin Precipitate.—The loss of its affinity for water is not the only change produced in a specific antibody globulin when it combines with antigen (bacteria, red cells, or dissolved protein). In addition to becoming denatured, and thus causing the flocculation of the antigen with which it has combined, it develops a marked avidity for complement (the hemolytic substance present in fresh serum), adsorbing it irreversibly (complement fixation). When the antigen-antibody complex is heated to 100° for a few seconds, this remarkable property is completely destroyed, presumably because of the heat-coagulation of the active film of denatured antibody (Eagle, 1929, 2).

If the mechanism of the precipitation of lipoid antigen by syphilitic serum is, as outlined in the preceding sections, exactly analogous to these specific antigen-antibody reactions, the precipitate formed should

possess the same complement-fixing properties as agglutinated red cells, bacteria, or a protein-antiprotein precipitate.

Antigen alone does not fix complement; nor does an adsorbed film of normal serum protein endow it with this property. As already shown by Wassermann (1921), and Ravenel and Dulaney (1925), however, the precipitate obtained with syphilitic serum fixes complement powerfully (Table VI, Row 1).

Protocol 6

x cc. of the washed lipoid-reagin precipitate were incubated with 0.4 cc. of 1:10 complement for ½ hour at 37°, and residual complement determined by a method already described (Eagle, 1929, 1). An individual experiment is given in detail.

Precipitate suspension, cc.	0.4	0.2	0.1	0.05	0.025	0.0125	0.006
Time for hemolysis of sensitized cells added after ½ hour, in seconds	>1800	>1800	>1800	360	135	105	90
Per cent complement fixed	>90	>90	>90	70	40	15	<10

0.4	cc. of complement	causes	hemolysis	in	90	seconds	
0.2	" "	"	"	"	150	"	
0.1	" "	"	"	"	480	"	
0.05	" "	"	"	"	900	"	

A series of experiments are summarized in Table VI, omitting similar technical details.

Even more interesting are the results in the second part of the table. A film of normal serum protein does not interfere with the specific reaction between the antigen particles and subsequently added syphilitic serum (reagin): the normal protein is only loosely bound and does not obscure the specific reacting groups of the lipoid. The lipoid-reagin precipitate, however, incubated with very strongly positive syphilitic serum, does not give any further reaction; the first incubation has covered the reacting groups of the lipoid with closely adherent reagin-globulin, so firmly bound as not to allow combination with more reagin (Row 3). Heating at 100° for a few seconds coagulates and destroys this complement-fixing film of protein without affecting the underlying lipoid. Their reacting groups once again free, the antigen particles give powerful fixation with the same syphilitic serum which was previously ineffective (Row 6).

The reagin film therefore differs from adsorbed normal protein not

only in its water-insolubility, and tendency to flocculate; but also in its ability to fix (adsorb) complement. Moreover, unlike normal protein, it attaches so firmly to the specific groups of the lipoid as to prevent any

TABLE VI

Complement-Fixing Properties of the Lipoid-Reagin Precipitate

Precipitate suspension, cc.	0.1	0.05	0.025	0.0125	0.006	0.003	0.0015	
Per cent complement fixed by x cc. of lipoid-reagin precipitate suspension	>90	60	35	20	10	<10	<10	
Per cent complement fixed by x cc. suspension + 0.4 cc. of 1:40 antigen	>90	60	35	20	10	<10	<10	No free reagin
Per cent complement fixed by x cc. suspension, + 0.1 cc. very strongly positive serum	>90	70	40	25	10	<10	<10	No free antigen (at most, minimal quantities)
Per cent complement fixed by x cc. of suspension, heated to 100°C. for 1 minute	0	0	0	0	0	0	0	Adsorbing reagin film destroyed by heat
Per cent complement fixed by x cc. heated suspension, + 0.4 cc. antigen 1:40	0	0	0	0	0	0	0	No free reagin
Per cent complement fixed by x cc. heated suspension, + 0.1 cc. very strongly positive syphilitic serum	>90	>90	>90	>90	>90	75	40	**Free antigen**

further combination with additional reagin. For lack of a better term, we may call this group of properties, induced in all antibodies by their combination with antigen, denaturation, remembering it to be quite distinct from the coagulation of a protein by heat.

IV. SUMMARY AND DISCUSSION

A. The lipoid antigen used in the serum diagnosis of syphilis, when colloidally dispersed in water, forms a relatively stable amphoteric suspension with predominantly hydrophilic properties. Although the colloidal particles flocculate at their isoelectric point (pH 1.9), in more alkaline reaction the negative surface potential prevents their cohesion and must be depressed to 1 to 5 millivolts before visible flocculation is obtained, indicating a very slight affinity between the colloidal particles. The amount of electrolyte necessary to depress this surface charge below its critical value decreases somewhat with increasing concentration of the sol, but is uniformly large: in a suspension containing 0.04 per cent lipoid, 1 M univalent and 1/40 M bivalent cation are the coagulation values.

B. In normal serum, hydrophilic protein is adsorbed, forming a protective film around the individual lipoid particles, with a corresponding change in the cataphoretic potential and the isoelectric point towards those of serum protein, the degree of shift depending upon the extent of the adsorbed film. The critical potential, however, is not affected, and the lipoid remains as stable away from its isoelectric point as in the absence of serum. The water-soluble film of unchanged protein is readily removed by washing, and does not prevent the subsequent combination of the underlying lipoid with the specific component of syphilitic serum.

C. When the lipoid antigen is added to syphilitic serum, in addition to this loose adsorption of normal protein it combines more or less irreversibly with a specifically altered fraction of the serum globulin (reagin), demonstrable in the washed precipitate both chemically and by sensitization experiments. Like adsorbed normal serum, it depresses the surface potential and causes a shift in the isoelectric point; but there the similarity ends. The reagin-globulin is rendered water-insoluble by its firm combination with the lipoid, exactly as any antibody is denatured upon combination with its specific antigen (bacteria, red cells, or dissolved protein). The hydrophobic films of reagin have five times as great an affinity for each other as the original lipoid surfaces; accordingly, the critical potential is raised from its original value of 1 to 5 millivolts to 10 to 15 millivolts, that of particles of de-

natured globulin or of any antigen-antibody complex, and relatively small quantities of electrolytes (at serum pH, cations) suffice to depress the stabilizing potential below this critical level, with resultant aggregation and flocculation. In brief, a specific globulin combines with the colloidal particles of the antigen, conferring upon them the unstable properties of a suspension of denatured protein.

Like the antibody film on bacteria, or red cells, and unlike normal adsorbed protein, the reagin globulin on the lipoid particle can adsorb ("fix") complement. When this protein film is destroyed by heat-coagulation, the complement-fixing property is lost; concomitantly, the specific groups of the lipoid having been freed from the closely adherent reagin, the antigen becomes again active, able to react with more syphilitic serum.

These changes in the properties of reagin globulin upon its combination with the lipoid antigen (denaturation) are in every sense analogous to those effected in any antibody by its specific antigen, and are probably due to the same, as yet unknown, factors. It has been suggested for bacterial and red cell "agglutinins" and protein "precipitins," that the groups of the antibody determining its specificity are also those which endow it with its hydrophilic properties; when these combine with antigen, residual free hydrophobic groups determine the surface properties of the complex. The same tentative hypothesis may be offered for the denaturation of reagin globulin by the lipoid antigen.

The complete analogy between the flocculation reactions for syphilis and the so-called specific reactions (bacterial and red cell agglutination; protein precipitation) suggests that like agglutinins, precipitins, etc., reagin globulin represents an antibody response to products of infection.

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STUDIES IN THE SEROLOGY OF SYPHILIS

II. The Physical Basis of the Wassermann Reaction

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(Received for publication, July 7, 1930)

A complete exposition of the theories which have been advanced to explain the Wassermann reaction would be beyond the scope of the present paper. The recent tendency is to regard it as a "colloidal phenomenon," which, in view of the fact that all the reagents are in colloidal solution, is a paraphrase of the problem rather than an explanation. Stern (1923, 1924) and Reiner (1929), finding that the addition of tannin to an antigen (organ-lipoid) suspension endows it with complement-fixing properties, have suggested that the Wassermann reaction is due to a similar dehydration of the antigenic particles by syphilitic serum. It is difficult to correlate such an hypothesis with the experimental fact that the complement-fixing properties of a biologically-sensitized antigen are completely destroyed at 100°C., while the antigen as such is thereby unaffected.

It has been shown (Northrop and de Kruif, 1922; Shibley, 1926; Mudd and Mudd, 1926, 1927; Eagle, 1929, 1930, 1) that the basic immune reaction involves the deposition of a film of denatured antibody globulin around the antigenic red cell, bacterium or foreign protein with which it has combined; and that both flocculation and complement fixation are secondary reactions. The former results from the discharge of this hydrophobic surface film of denatured antibody by electrolyte; the latter is an adsorption of complement by this same globulin film. The cause of the denaturation which endows the antibody globulin with these hydrophobic and complement-avid

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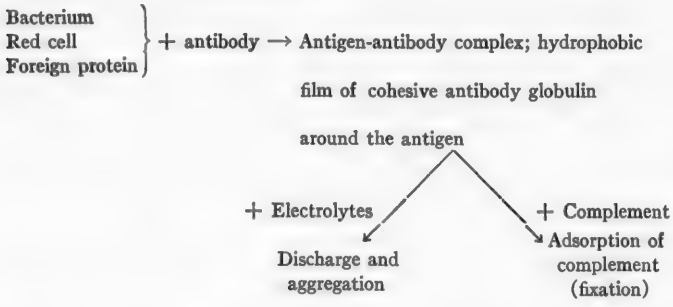
properties is as yet unknown; a tentative hypothesis was suggested which need not be discussed here.



The flocculation of lipoid antigen by syphilitic serum is exactly analogous to these antigen-antibody reactions (Eagle, 1930, 3). Reagin globulin is denatured upon its combination with the lipoid antigen, forming a hydrophobic sensitizing film with a critical potential of 10 to 15 millivolts, as contrasted with 1 to 3 millivolts for the original antigen. NaCl N/15 suffices to depress the surface charge below this critical level, allowing the cohesion of the sensitized particles, a cohesion primarily of the reagin globulin film, and only incidentally of the underlying lipoid antigen.

It remains to show that, as in the case of the so-called specific reactions, the same globulin film which causes flocculation also determines complement fixation. At first sight, the high degree of correlation between the Wassermann and flocculation tests (Georgi, 1919; Mason, 1920; Sahlmann, 1922; Kritschewsky, 1928) would seem to establish our thesis. However, scattered evidence to the contrary in the literature (Wendlandt, 1920; Gloor and Klinger, 1920; Nathan, 1922), and the occasional descrepancies between the two types of test necessitate an empirical proof that precipitating and complement fixing reagin are identical.

1. a. If the Wassermann reagin is identical with the substance in syphilitic serum which is responsible for flocculation, it should be associated with the same fraction of serum protein, the globulin. The literature bearing on this point is quite conflicting.

Felke (1920–1921) thinks that the active substance is contained primarily in the albumen fraction; yet his own protocols show that removal of the globulin caused a certain proportion of positive sera to become negative. Gloor and Klinger (1920) state unqualifiedly that although the globulin fraction determines flocculation (Sachs-Georgi reaction), the albumen is the carrier of Wassermann reagin. On the other hand, Kapsenberg (1921) finds the globulin of a positive serum to be positive always, while the albumen fraction if positive, is weakly so. In a later paper (1924) he comes to the conclusion that this residual reagin in the albumen is due to an incomplete removal of globulin.

This source of error, which vitiates the results of Felke and of Gloor and Klinger, has been emphasized by Sahlmann (1922). Both the globulin precipitate and the supernatant fluid may be positive after the fractionation of syphilitic serum with CO_2 or HCl; but since only a portion of the globulin (the so-called euglobulin) is thereby precipitated, no conclusion can be drawn as to the distribution of reagin between globulin and albumen. The experiments of Stern (1923, 1924) seem definitive. Such procedures as simple dialysis, or precipitation by CO_2 or HCl, cause an incomplete separation of globulin and therefore of reagin; but when all the globulin is precipitated, as by electrodialysis, all the Wassermann reagin is concentrated in the sediment, and the supernatant albumen is completely negative.

b. Another objection to the globulin theory of Wassermann reagin was raised by Skrop (1923). The active substance was stated to have a positive charge, migrating to the cathode in an electrical field, while globulin at serum pH ionizes as Na^+ globulinate$^-$; the globulin ion being negatively charged and migrating to the anode. These experimental data have been vigorously discredited by Stern (1923). What was supposed to be reagin concentrated at the cathode was actually acid (pH 3.8 to 6.1) which accumulates during dialysis; the supposed complement-fixation it caused was a non-specific destruction. Furthermore, the fact that negatively charged particles adsorb reagin does not prove it to be positively charged: both negatively and positively charged substances may be adsorbed by negatively charged particles (*e.g.*, Kaolin).

Indeed, upon passing syphilitic serum through a series of 4 dry Berkefeld filters, we found that there had been a 30 per cent decrease of reagin-content, presumably due to adsorption by the negatively charged particles of the filters: but there was a parallel decrease in serum protein (25 per cent), proving that opposite charges are not a prerequisite for adsorption, and suggesting that serum protein is the carrier of the substance determining the reaction.

c. The constant association of the Wassermann reagin with serum globulin does not, of course, establish their identity. However, every effort to separate the two has failed. Forssman (1921) has stated that if the globulin precipitate obtained from syphilitic serum by acidification with acetic acid is redissolved in NaCl, and the solution shaken with ether, it becomes Wassermann negative; but that if the ether extract is allowed to evaporate onto this solution, it again becomes Wassermann positive. Apparently, this proves that reagin is an ether-soluble lipoid merely carried down with the globulin. However, his whole case is given away by the fact that not only the extract, but even normal ether can restore the positivity of the extracted globulin solution.

Upon repeating his experiments it is found that when a solution of serum globulin is shaken with ether, most of the protein is coagulated, probably due to adsorption and denaturation at the ether-water interfaces in the emulsion. The disappearance of the positive reaction is therefore due to an irreversible coagulation of the reagin-globulin, and not to an extraction of a hypothetical reagin-lipoid. When ether is layered onto this suspension of denatured globulin and allowed to evaporate, one obtains a highly anticomplementary solution, due to acids contained in the ether. The supposedly restored positive Wassermann is in reality a non-specific destruction of complement by acid impurities in the ether.

To summarize, Wassermann reagin is constantly associated with the serum globulin, confirming its identity with the substance which causes the flocculation of antigen. Further evidence is given in the following sections.

2. In every positive Wassermann reaction there is microscopically visible aggregation of the lipoid particles into small aggregates of two or three particles (Jacobsthal, 1911). There are no large visible or sedimenting clumps solely because the antigen is used in too small quantities to give optically visible aggregates.

3. It is well known (Jacobsthal, 1911; Ravenel and Dulaney, 1925; Wassermann, 1921) that the antigen lipoid suspension alone does not fix complement, while the lipoid-protein aggregates obtained by adding this suspension to syphilitic serum fix complement powerfully.

4. It is also known that heat, which would coagulate the film of globulin, destroys this acquired complement-fixing property (Stern,

1929). Simultaneously, the antigen particles regain their ability to fix complement with syphilitic serum, a property previously masked by the surface film of denatured reagin with which they had already combined (Paper I of this series; Table VI).

5. The fact that certain conditions will inhibit complement fixation without affecting flocculation, or *vice versa*, does not disprove the identity of the substance causing the two. The basic reaction in any serological test for syphilis is always the same: particles of lipoid antigen combine with reagin globulin, which is deposited as a film of denatured protein around the underlying lipoid. The subsequent aggregation of these sensitized particles due to the hydrophobic properties of this protein film and the subsequent adsorption due to its avidity for complement midpiece are entirely different physico-chemical processes, with different optimal conditions. Complement fixation takes place at pH 5.8 to 8.2, and at a NaCl concentration of 0.07 to 0.25 N, with inhibition on either side of this optimal range; it is only slightly affected by volume and shaking, is completely suppressed by relatively slight concentrations of bivalent cations, and because of the thermolability of complement, must be carried out at $<40°$C. (Eagle, 1929, 3). The cohesion of the protein-coated particles on the other hand takes place most rapidly at pH 3.5 ± 0.5, is enormously accelerated by shaking, by decrease in total volume, by bivalent cations or by an increase in temperature up to 56°C.

One can therefore pick any number of variables which would have opposite effects upon the two types of reaction: but obviously this is due to differences in the types of reaction and does not connote a different causal factor.

6. Contrary to the statement of Gloor and Klinger (1920), compement-fixing reagin is completely removed by the antigen.

If an excess of antigen is added to syphilitic serum, and the precipitate removed by centrifugation (or by Berkefeld filtration) it is found that the supernatant fluid no longer gives a positive flocculation test upon a second addition of antigen; moreover, it no longer fixes complement, *i.e.*, it gives a negative Wassermann test. Provided only that sufficient antigen is used to combine with all the reagin, more than 99 per cent of the complement-fixing substance is concentrated in the precipitate (Table I, Protocol 1).

Protocol 1

To x cc. of strongly positive syphilitic serum were added 1/6 x cc. of an alcoholic solution of cholesterinized antigen and NaCl N/7 to 2 x cc. The precipitate was removed by a) prolonged centrifugation and b) Berkefeld filtration, and the complement fixing properties of the original serum, precipitate and supernatant fluid determined by a method described elsewhere (Eagle, 1930, 2). The unit of Wassermann reagin is taken as that necessary to give 75 per cent complement fixation: the titre is expressed as the dilution of serum containing 1 unit.

TABLE I

	0.4 cc.	0.2 cc.	0.1 cc.	0.05 cc.	0.025 cc.	Units reagin per cc.	Flocculation test
Serum 1:2, per cent fixation (x cc. + 0.4 cc. complement 1:10 + 0.4 cc. antigen).....	>90	>90	>90	80	50	22	++++
Supernatant, after centrifugation, per cent fixation (x cc. + complement 1:10; anticomplementary determination).....	50	25	10	0	0	2 units anticomplementary	0
Supernatant after centrifugation, per cent fixation (x cc. + complement + antigen; anticomplementary + reagin).....	50	25	10	0	0	2 units anticomplementary + reagin	0
Supernatant after centrifugation; per cent fixation by free reagin.....	<10	0	0	0	0	0 reagin units	0
Berkefeld filtrate per cent fixation x cc. + complement + antigen.....	(20) (?)	0	0	0	0	0	0

As seen in Table I, the supernatant fluid after centrifugation contains less than 1/20 as much free reagin as the original serum. It is somewhat anticomplementary, due to the fact that small clumps of antigen sensitized with reagin are not completely carried down; but a further addition of antigen does not increase the degree of complement fixation, *i.e.*, there is no free reagin. Berkefeld filtration removes these

sensitized antigen particles completely, with the result that the clear filtrate is no longer anticomplementary, and contains no demonstrable reagin.

SUMMARY AND DISCUSSION

The substance in syphilitic serum which is responsible for the Wassermann reaction, like that which determines the diagnostic flocculation tests, is associated with the globulin fraction of serum.

Every positive Wassermann is accompanied by microscopic (or submicroscopic) aggregation, which is not an essential feature of the reaction; conversely, after every positive flocculation test, the washed precipitate will fix complement. An excess of antigen removes both flocculating and complement-fixing substances completely (>95 per cent). Heating the lipoid-reagin precipitate to 100° for 1 minute destroys the sensitizing film of reagin globulin; the avidity for complement disappears simultaneously.

Both the flocculating and complement-fixing properties of syphilitic serum are therefore determined by the same substance, a specifically altered fraction of the serum globulin, reagin. The Wassermann reaction is thus entirely analogous to complement fixation by any antigen-antibody complex. The same film of denatured serum globulin which sensitizes the antigen particles, whether red cells, bacteria, protein, or colloidal lipoid particles, to discharge and aggregation by electrolytes, also endows them with an avidity for complement.

The pathogenesis of reagin will be discussed in a forthcoming paper.

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STUDIES IN THE SEROLOGY OF SYPHILIS

III. Explanation of the Fortifying Effect of Cholesterin upon the Antigen as Used in the Wassermann and Flocculation Tests

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Soon after the discovery that an alcoholic extract of normal tissue could be used in the serum diagnosis of syphilis (Landsteiner, Muller and Pötzl, 1907), it was found by Browning, Cruikshank and Mackenzie (1910) that small quantities of cholesterin added to such an extract increased its sensitivity markedly. Although its use both in the complement fixation and flocculation tests (Kahn, Sachs-Georgi, Meinicke, Hinton, etc.) has since become almost universal, there is as yet no satisfactory explanation of its effect.

It is certainly not in itself antigenic (Protocol 1 and Table I): 100 times as much cholesterin as the fixing quantity of antigen has no effect with syphilitic serum. As Maltaner (1930) recently showed, its activity is not to be ascribed to traces of activated ergosterol: weight for weight, the latter is no more effective than pure cholesterol. Most of the text-books speak of an obscure physico-chemical effect. The explanation of its action is the subject of the present paper.

Protocol 1

1 cc. of an alcoholic extract of beef heart containing 1½ per cent total solid was dropped with shaking into 40 cc. NaCl N/7. To decreasing quantities of this colloidal lipoid suspension were added 0.4 cc. of 1:10 guinea pig complement, 0.4 cc. of 1:4 strongly positive syphilitic serum and NaCl N/7 to 1.2 cc. After 4 hours at ice box temperature and ½ hour at 37°C., 0.8 cc. of strongly sensitized sheep cells were added to each tube, and the time for complete

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hemolysis determined in seconds. The degree of complement fixation was calculated from hemolysis time by a method already described (Eagle, 1929, 1; 1930, 1).

An exactly similar experiment was carried through at the same time with the same reagents, using 1½ per cent cholesterin instead of antigen, diluted with water instead of saline. An individual experiment is summarized in Table I.

I. Cause of the Stable Dispersion of Cholesterin in the Presence of Antigen

Both the antigen and cholesterin are water-insoluble and alcohol-soluble, but they differ markedly in their affinity for water. The anti-

TABLE I

Cholesterin as Such Has No Effect with Syphilitic Serum

cc. of 1:10 dilution of 1½ per cent antigen (or cholesterin)	0.4	0.2	0.1	0.05	0.025	0.0125	0.006	0.003
Per cent fixation by x cc. antigen + 0.1 cc. syphilitic serum	>90	>90	>90	>90	>90	90	50	30
Anticomplementary control of antigen, per cent destruction	25	10	0	0	0	0	0	0
Actual fixation by antigen	>90	>90	>90	>90	>90	90	50	30
Apparent fixation by x cc. cholesterin + 0.1 cc. syphilitic serum, per cent.	25	10	0	0	0	0	0	0
Anticomplementary control cholesterin, per cent destruction	25	10	0	0	0	0	0	0
Actual fixation by cholesterin, per cent	0	0	0	0	0	0	0	0

gen, although insoluble as such, can be readily peptized by dropping the alcoholic solution into an excess of water. The colloidal solution obtained is not only stable, but is remarkably hydrophilic for a water-insoluble substance, remaining colloidally dispersed until the surface potential of the constituent particles has been reduced to 1 to 5 millivolts (0.75 to 1.5 N NaCl), when they cohere to form unstable aggregates. Although the suspension is optically homogeneous, these discrete particles can be seen by dark field illumination as minute refractile bodies in active Brownian motion.

An alcoholic solution of cholesterin on the other hand cannot be so peptized. Dropped into water, needle crystals of cholesterin form immediately, which despite their very high mutually repellent surface

charge, quickly coalesce to form unstable aggregates. The difference in affinity to water can be expressed as the ratio of their critical potentials:

$$\frac{> 100 \text{ (cholesterin)}}{1\text{–}5 \text{ (antigen)}}$$

However, when the cholesterin is added to an alcoholic solution of antigen and the mixture is dropped into an excess of water, instead of obtaining a precipitate of cholesterin and a colloidally dispersed antigen, both remain colloidally dispersed in an optically homogeneous solution. Corresponding to its greater opalescence, there are more minute particles to be seen, of somewhat larger size than those formed by antigen alone, but no cholesterin as such. Obviously, each particle contains antigen and cholesterin, and the complex is more efficient in both complement fixation and precipitation than the antigen particle alone.

To hypothecate a chemical combination as the explanation of the peptization of the cholesterin would be begging the question. When one drops an alcoholic solution of cholesterin into an aqueous colloidal suspension of antigen, it precipitates just as it would in pure water. There is certainly no reaction between the two substances in alcoholic solution, and they can be readily dissociated from their complex colloidal sol. Nor would such an hypothesis explain the greater sensitivity of the lipoid-cholesterin sol in the diagnostic reactions unless we introduce as another assumption that the hypothetical compound is more reactive than the lecithin antigen alone. We find an analogy in the detergent action of soap upon fatty acids, silica, etc. The soap is adsorbed by the particles of dirt, forming a hydrophilic film which allows them to pass into the solution, and prevents their cohesion (Freundlich, 1922). Similarly, we can hypothecate that when the alcoholic antigen-cholesterin solution is dropped into water, the natural tendency of the cholesterin molecules to cohere is prevented by the fact that, as submicroscopic aggregates form, they adsorb the lecithin antigen; this would form a protective film, preventing any further aggregation and allowing the particles to remain colloidally dispersed. Each particle seen microscopically would thus contain both antigen and cholesterin, not in any haphazard distribution, but in a definite

arrangement: a nucleus of cholesterin surrounded by an adsorbed protective antigen surface of indeterminate thickness. (See Fig. 2.)

The forces determining this adsorption would be those of interfacial tension. If

$$\rho \text{ cholesterin-water} > \rho \text{ cholesterin—antigen} + \rho \text{ antigen-water},$$

that is, if the surface tension of cholesterin against water were greater than the sum of the antigen-water and cholesterin-antigen interfacial tensions, then the antigen would be adsorbed, since the total free surface energy would be thereby diminished. Unfortunately, there are no methods for measuring solid-solid or solid-liquid interfacial tensions. Qualitatively, the complete insolubility of cholesterin, as contrasted with the ease of peptizing antigen, as well as the known low surface tension of lecithin against air (Price and Lewis, 1929) suggest that ρ Chol.—H_2O > ρ Ant.—H_2O, but we have no indication of the magnitude of ρ Ant.—Chol.

There are, however, several other methods of verifying the suggested explanation. If surface-active antigen actually causes the stable dispersion of cholesterin by forming a protective film around minute particles of cholesterin, preventing their further aggregation, then (1) the surface properties of the individual antigen-cholesterin particles should be those of the antigen, the properties of cholesterin being completely masked by the superimposed film; (2) any substance with the same physical properties as antigen should cause a similar dispersion of cholesterin in water; (3) any substance of the same physical properties as cholesterin should be as readily peptized by the lipoid antigen. The empirical data follow.

A. Surface Properties of the Antigen-Cholesterin Particles.—

1. Isoelectric Point.—The isoelectric point of the lecithin antigen is pH 1.9 (Eagle, 1930, 2), corresponding to the fact that it contains both phosphoric acid and fatty acid radicals, and a comparatively weak organic base. Although that of cholesterol is usually given as pH 3–4 ±, it is difficult to understand from its chemical structure why it should be amphoteric, containing as it does only one active group, an alcohol. Accordingly, it was not surprising to find that of six different samples, no two had the same cataphoretic isoelectric point

(Table II). Presumably, therefore, the so-called isoelectric point of cholesterin is due to an associated impurity (glycocholl, etc.).*

The point of interest in the present connection, however, is that no matter what the original isoelectric point of the particular sample of cholesterin, that of the particles of an antigen-cholesterin sol is always the same, pH 1.9 ± 0.2, the isoelectric point of antigen alone; indicating that the surface of the particles is not a mosaic of cholesterin and antigen, as it would be in a haphazard distribution, but antigen only, surrounding a core of cholesterin. (Table II.)

TABLE II

Showing that the isoelectric point of the particles of an antigen-cholesterin sol is independent of that of the cholesterin, depending solely upon the antigen surface.

Manufacturer	Isoelectric point of cholesterin particles	Isoelectric point of particles of antigen-chol. sol (ant.:chol. ratio = 1/1)
Hollister	3.3	1.8
Pfanstiehl	2.1	1.9
Eimer and Amend	3.2	1.95
Merck	3.4	2.0
Laboratory Spec. 1, from gallstones	2.5	1.8
" " 2, " "	3.6	1.8

2. Critical Potential and Coagulation Value.—An alcoholic solution of antigen alone when dropped into water forms a very stable suspension at any pH > 1.9. Thus, at pH 7.4 the critical potential, the minimum compatible with stability, is 1 to 3 millivolts, a very low value for a water-insoluble substance. Its flocculation value, *i.e.*, the amount of electrolyte necessary to reduce the surface potential below this critical value, is 0.5 to 1.5 N for univalent cations. The rather wide limits given are due to the fact that the stability of the sol varies with its concentration (Eagle, 1930, 2).

Cholesterin on the other hand, despite its very high cataphoretic potential (>100 millivolts) flocculates even in water. If the particles

* The extremely low buffer value of an alcoholic solution of cholesterin as contrasted with one of truly amphoteric antigen, is further proof that its isoelectric point is due to an impurity.

of the antigen-cholesterin sol consisted of the two substances in a haphazard distribution, the surface would consist of a mosaic of antigen and cholesterin, and its surface properties would be intermediate between the two. Instead, even with 2 parts of cholesterin to one of antigen, the critical potential of the complex antigen-cholesterin sol is still 1 to 5 millivolts, the value of pure antigen, instead of about 70, as it would be if the particles contained antigen and cholesterin in a statistical distribution (Protocol 2 and Table III).

Protocol 2

Alcoholic solutions of antigen (solid content 1.6 per cent) containing increasing quantities of cholesterin were shaken with an equal volume of NaCl N/7. The suspensions were centrifuged and made up in H_2O to their original volume, forming suspensions containing 0.8 per cent antigen lipoid plus varying quantities of added cholesterin. 0.2 cc. of each suspension were added to 3.7 cc. of NaCl of varying concentration, and brought to approximately pH 7.4 with 0.1 cc. of phosphate buffer M/15. The figures in the body of Table III represent degrees of flocculation after 24 hours at room temperature. The slight decrease in stability with added cholesterin is due entirely to the increased number of particles, those most readily flocculated.

The only explanation for this anomalous finding is that cholesterin is concentrated in the center of the particles as its core, antigen forming a surface film which determines the surface properties of the complex particle.

TABLE III

Showing that cholesterin does not appreciably affect the coagulation value or critical potential of the antigen sol (pH 7.4).

Composition of alcoholic solution	Cholesterin + antigen	Flocculation after 24 hours							Isoelectric point	Coagulation value (NaCl)	Critical potential, millivolts
		N/1	N/2	N/4	N/8	N/16	N/32	0			
Antigen lipoids 1.6%	0	0	0	0	0	0	0	0	1.9	N/1	<2
Antigen lipoids 0.8% + cholesterin 0.4%	1/2	±	0	0	0	0	0	0	1.9	N/1	<2
Antigen lipoids 0.4% + cholesterin 0.4%	1/1	4	±	0	0	0	0	0	2.0	N/2–N/1	<2
Antigen lipoids 0.2% + cholesterin 0.4%	2/1	4	2	0	0	0	0	0	1.7	N/2–N/1	2–3
Antigen lipoids 0 + cholesterin 0.4%		4	4	4	3	3	3	3	3.6	0	>100

B. Peptization of Other Water-Insoluble Substances by Antigen.—The explanation suggested for the formation of the stable lipoid-cholesterin sol implied as a second and necessary corollary that any substance of the same physical properties as cholesterin should be similarly peptized by antigen. The most diverse types of substances were tested, all water-insoluble, alcohol soluble and all flocculating either immediately or within a few hours when the alcoholic solution is dropped into an excess of NaCl N/7. In the majority of cases the presence of antigen in the alcoholic solution allowed the substance to

TABLE IV

Effect of Antigen upon the Stability of a Series of Alcohol-Soluble, Water-Insoluble Substances

Substance	Alcoholic solution dropped into NaCl N/7	Alcoholic solution + antigen dropped into NaCl N/7
Phenyl salicylate	Precipitate	Stable opalescent sol
Gums: Mastic, elemi, copal, sandarac, benzoin, etc.*	"	"
Palmitic acid	"	Precipitate
Benzophenone	"	Stable opalescent sol
Ethyl stearate	"	"
α-Naphthonitrile	"	"
Triphenylphosphine	"	"
Cetyl alcohol	"	Precipitate
p-Nitrobromobenzene	"	Stable opalescent sol
Benzil	"	"
etc., etc.		

* The precipitate obtained may be a resinous impurity.

be colloidally dispersed irrespective of its chemical nature, corroborating the hypothesis that the hydrophilic antigen forms a protective film around the hydrophobic particles in *statu nascendi*, preventing their further aggregation into sedimenting clumps (Table IV).

For all these substances, then,

$$\rho \text{ substance-water} > \rho \text{ substance-antigen} + \rho \text{ antigen-water};$$

as in the case of cholesterin, their interfacial tension against water exceeds that of the two surfaces formed by the adsorption of the lecithin

antigen. When the alcoholic solution (of antigen + x) is dropped into water, minute particles of x therefore adsorb antigen while they are still $<1\mu$ in diameter. The complex particles formed have a surface of hydrophilic antigen, and are as stable as antigen particles of the same size.

C. Peptization of Cholesterin by Substances Other Than "Antigen."—Conversely, not only the alcoholic extract of beef heart, but any similar substance will cause the colloidal dispersion of cholesterin, again indicating that the phenomenon is determined, not by a specific chemical affinity between antigen and cholesterin, but by their physical properties. Thus, if cholesterin is added to an alcoholic extract of dried milk (or of egg yolk) and the mixture dropped into an excess of water or saline, one obtains a stable milk (egg) lipoid-cholesterin sol. Similarly, an alcoholic extract of any animal tissue will cause a stable colloidal dispersion of aggregates of cholesterin before they become sufficiently large to sediment. Indeed, an aqueous solution of any substance with a low interfacial tension against both cholesterin and water should have the same effect. To test this, an alcoholic cholesterin solution was dropped into solutions of serum, Na-oleate, Na glycocholate, and Na taurocholate in NaCl N/7, all surface-active substances of varying efficiency. In all four, there was obtained an opalescent, but stable colloidal suspension of cholesterin, contrasting with the coarse rapidly sedimenting aggregates obtained in saline alone. Here again we may write that

$$\rho \text{ cholesterin-water} > \rho \text{ cholesterin-serum} + \rho \text{ serum-water}$$
$$[\text{or}] > \rho \text{ cholest.—Na-oleate, etc.} + \rho \text{ Na-oleate-water, etc.}$$

To summarize, since the particles of an antigen-cholesterin sol have the same isoelectric point, critical potential and coagulation value as antigen alone; since any similarly surface active substance (egg or milk-lipoid, etc.) can be substituted for the beef heart lipoid antigen as the peptizing agent; and since antigen will cause a similar colloidal dispersion of any hydrophobic alcohol-soluble substance like cholesterin: we conclude that antigen causes the colloidal dispersion of cholesterin by forming a protective film around minute aggregates of cholesterin before they exceed the limits of colloidal stability, endowing them with its own stable surface properties, and preventing their further aggregation into sedimenting clumps.

The phenomenon is therefore analogous to the detergent action of soap. The adsorption of a surface-active hydrophilic substance (antigen, soap) by minute particles of a hydrophobic substance (cholesterin, dirt) allows the latter to form a stable colloidal suspension in water. The difference between the two is that the initially large particles of dirt are peptized by friction, and adsorb the soap as they break off; while the cholesterin, beginning as a molecularly dispersed alcoholic solution, would form very large unstable aggregates in water were it not for the fact that the tiny particles adsorb antigen before they become sufficiently large to sediment.

II. Physical Properties of the Antigen-Cholesterin Sol, as Contrasted with Antigen Alone

As already shown, the surface properties of the individual particles in the complex sol are determined by the adsorbed antigen surface; their isoelectric point and critical potential are therefore approximately the same as those of particles of pure antigen. There is, however, a very great difference in the degree of dispersion.

In any sol, the size of the particles varies widely. When an alcoholic solution of antigen alone is dropped into an excess of saline, it forms a highly dispersed colloidal solution in which only a few of the particles are within the limits of dark field visibility, probably less than 1/10. The remainder are even smaller, probably $<0.05\mu$ in average diameter. Corresponding to the small average size of the particles, the turbidity of the suspension is very slight. However, the addition of relatively small quantities of cholesterin to the antigen causes an enormous increase in the number of particles visible by dark field illumination. Thus, 1 part of cholesterin to 3 of antigen increases the number of visible particles twenty-fold, sixty times more than a similar quantity of antigen. Not only the number, but the average size of these visible particles increases *pari passu* with the amount of added cholesterin, as does the turbidity (Fig. 1). Clearly, when the cholesterinized antigen is dropped into saline, the cholesterin aggregates very rapidly; before an adequate protective cover of antigen can be adsorbed, most of the particles exceed in size the largest of these formed by antigen alone. And since the actual mass of the cholesterin is very small, these larger antigen-cholesterin particles must be obtained at the ex-

pense of the very small submicroscopic particles formed in the absence of cholesterin (Fig. 2). The net result is therefore a coarsened dispersion state of antigen, intermediate between the finely dispersed sol formed by antigen alone and the large visible clumps formed by cholesterin.

As the proportion of cholesterin to antigen in the alcoholic solution

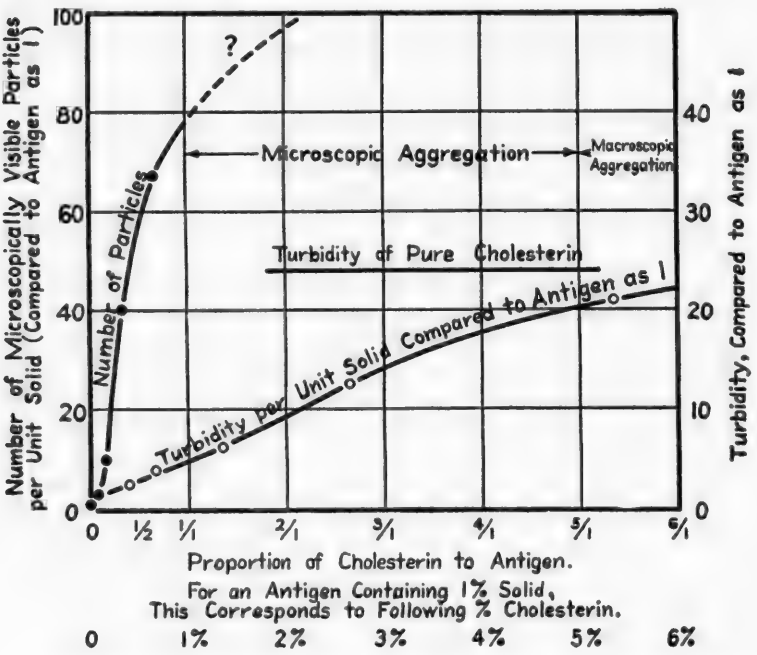

FIG. 1. Effect of cholesterin upon physical properties of the antigen sol (see Table V).

is increased (Table V), the microscopically visible particles in the aqueous sol become more and more numerous, and of larger average size; but they remain discrete until the cholesterin-antigen ratio is about 1/1. Any further increase in the proportion of cholesterin results in the formation of tiny microscopic aggregates; however, these do not become sufficiently large to sediment or be visible microscopically

until the alcoholic solution contains 5 to 7 parts of cholesterin to 1 of antigen. At this critical ratio, the sol is no longer homogeneous and opalescent, but becomes cloudy. With even more cholesterin, there is progressively more rapid flocculation and sedimentation of coarse grossly visible aggregates.

Paralleling the coarsened dispersion state of the antigen caused by cholesterin, the turbidity of the sol per unit solid increases, approaching as its maximum value that of a suspension of pure cholesterin. It should be noted that as little as 1 part of antigen to 8 of cholesterin causes a significant change in the turbidity, *i.e.*, of its degree of dispersion, in keeping with the fact that it forms a thin surface film. These data are all summarized in Protocol 3 and Table V.

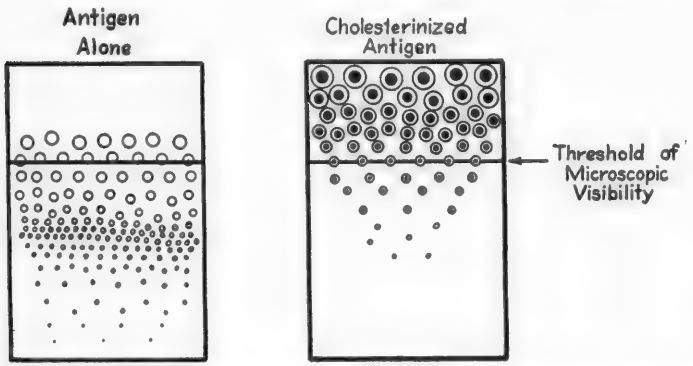

FIG. 2. Diagrammatic representation of effect of cholesterin upon particle number and size in an antigen sol (solid black core indicates cholesterin).

Protocol 3

A series of tubes with varying proportions of antigen and cholesterin were set up as outlined in Table V, and dilutions made by dropping into saline. The number of particles were counted in the Petroff-Hausser counting chamber (1/50 mm. deep) by dark field illumination. The Brownian motion of the particles made counting difficult, and the figures given are necessarily approximate.

Turbidity measurements were made roughly by determining the degree to which each suspension had to be diluted in order to approximate the turbidity of the pure antigen sol. The results are summarized in Table V and Figs. 1 and 2.

TABLE V

Effect of Cholesterin upon the Physical Properties of Antigen Sol

Antigen (1.5%), cc.	0.4	0.4	0.4	0.4	0.4	0.4	0.2	0.1	0.05	0.025	0.0125
Cholesterin (0.5%), cc.	0	0.1	0.2	0.4	0.8	0.8	0.8	0.8	0.8	0.8	0.8
Cholesterin (1%), cc.											
Alcohol	1.6	1.5	1.4	1.2	0.8	0.8	1.0	1.1	1.15	1.2	1.2
Ratio of cholesterin to antigen	0	1/12	1/6	1/3	2/3	4/3	8/3	16/3	32/3	64/3	128/3
Mg. solid per cc.	3	3.25	3.5	4	5	7	5.5	5.75	4.37	4.18	4.09
Dilution prepared in NaCl N/7	1:10	1:10	1:100	1:100	1:1000	1:1000	1:400	1:400	1:400	1:400	1:400
Appearance of aqueous dilution	Stable sols of increasing opalescence: no microscopic aggregation					Microscopic aggregation but stable. No visible flocculation in 24 hours			Increasing degrees of microscopic aggregation. Increasing velocity of macroscopic flocculation as proportion of cholesterin is increased		
Microscopically visible number of particles in 1/20,000,000 cc.	4	13	5	25	5						
Particles per cc. of alcoholic solution	8×10^8	3×10^9	10^{10}	5×10^{10}	10^{11}						
Particles per mg. solid	3×10^8	10^9	3×10^9 (?)	1.2×10^{10}	2×10^{10}						
Turbidity of solutions per unit solid, referred to antigen as 1	1	1.3	1.5	2.4	4	6.2	12.5	18	24	24	24

III. Explanation of the Effect of Cholesterin in the Wassermann Reaction

Complement fixation in the Wassermann reaction is an adsorption of complement midpiece by the same film of denatured reagin-globulin which sensitizes the particle to flocculation by electrolyte (Eagle, 1930, 3). The degree of fixation increases with the amount of antigen used (Table I), and since the reaction takes place at the surface of the particles, this is the equivalent of saying that it increases with the total surface area.

A priori, this would imply that the more finely divided a given quantity of antigen, the more efficient is the resulting sol in complement fixation: for surface increases with progressive subdivision. Actually, however, as the following experiment shows, within the range covered by the particles of antigen and antigen-cholesterin sols, a coarsely dispersed antigen dilution is much more sensitive in complement fixation, despite the fact that it has less surface, confirming the findings of Kermack and Spragg (1929).

If an alcoholic solution of antigen is dropped into an excess of NaCl N/7, it forms a highly disperse, faintly opalescent sol in which but few particles are visible by dark field examination. But if the saline is dropped into the antigen, keeping the quantities the same, a much more turbid suspension is obtained (Sachs and Rondoni, 1909) containing about 20 times as many visible particles, and correspondingly fewer small ones. The total surface area of this coarse water-into-antigen sol is much less, yet it is about twice as efficient in complement fixation with syphilitic serum (Protocol 4 and Table VI). Per unit surface, the larger particles are therefore at least 5 times as efficient.

Protocol 4

1 cc. of an antigen containing 1½ per cent solid matter was dropped into 39 cc. NaCl N/7. The transparent, very slightly opalescent sol contained 1 to 2 particles per 1/20,000 sq. mm. (2 to 4 $\times$ 10^7 per cubic centimeter) as counted by dark field illumination in the Zinsser-Hauser apparatus. When 39 cc. of NaCl N/7 were added with shaking to 1 cc. of the same antigen a markedly opalescent sol was obtained containing 4 to 6 $\times$ 10^8 visible particles per cubic centimeter, an increase of 20 fold. To x cc. of each antigen were added 0.4 cc. of guinea pig complement 1:10, 0.1 cc. of a strongly positive syphilitic serum and NaCl N/7 to 1.2 cc. The degree of fixation after 4 hours at ice box and ½ hour at 37°C. was determined by a method already described (Eagle, 1930, 1). The results are summarized in Table VI.

The fact that cholesterin causes just such a coarse dispersion of the antigen when the mixture is dropped into saline, as compared with the finely dispersed sol formed by antigen alone, completely explains the greater efficiency of the cholesterinized antigen in the Wassermann reaction.

The basic cause of this greater efficiency of large particles is of only theoretical importance. There are two possibilities: 1) either the larger antigen particles have a much greater avidity for reagin, or 2) having combined with antigen, the film of denatured reagin is a more efficient adsorbent for complement when it is spread around large particles. As shown by the following experiment, the first is the correct explanation.

TABLE VI

Contrast in the Complement-Fixing Efficiency of Antigen-into-Water and Water-into-Antigen Sols of the Same Solid Content

Antigen cc. of 1:40 dilution	0.4	0.2	0.1	0.05	0.025
Per cent complement fixed by x cc. water-antigen sol + 0.1 cc. of same positive serum	>90 (4+)	>90 (4+)	80 (4+)	45 (0)	25 (0)
Per cent complement fixed by x cc. antigen-water sol + 0.1 cc. positive serum	80 (4+)	70 (3+)	40 (0)	20 (0)	10 (0)

Decreasing quantities of a strongly positive serum were incubated for 1 hour with 0.4 cc. of a) 1:40 antigen, b) 1:40 cholesterinized antigen. At the end of this time, an additional 0.1 cc. of 1:40 cholesterinized antigen was added to all the tubes. After 15 minutes, 0.4 cc. of complement was added to each tube, and the degree of fixation determined after ½ hour at 37° by a method described elsewhere (Eagle, 1930, 1).

The combination between antigen and reagin is an irreversible one. The second dose of antigen could therefore combine only with free reagin not previously bound. As seen in Table VII, 75 per cent of all the reagin remains free after 2 hours' incubation with cholesterin-free antigen: the subsequent addition of as little as 0.1 cc. binds almost all this free reagin within 15 minutes, as shown by the degree of com-

plement fixation. Cholesterinized antigen, on the other hand, leaves almost no reagin free: accordingly, subsequently added antigen has practically no effect upon the degree of complement fixation.

TABLE VII

Cholesterinized Antigen Has Greater Avidity for Reagin

1:8 Syphilitic serum, cc.	0.4	0.2	0.1	0.05	0.025	Interpretation
a) 0.4 cc. antigen (no cholesterin) 75 minutes; per cent fixation	50	25	—	—	—	Weak fixation by cholesterin free antigen
b) As in a); 0.1 cc. cholesterinized antigen added after 60 minutes; per cent fixation	>90	70	40	—	—	Reagin left free by non-cholesterinized antigen bound by supplementary cholesterinized antigen
a) 0.2 cc. cholesterinized antigen 75 mins.; per cent fixation	>90	80	50	25		Strong fixation by cholesterinized antigen
b) As in a); 0.4 cc. cholesterinized antigen added after 60 mins.; per cent fixation	>90	80	50	25		No reagin left free by cholesterinized antigen

TABLE VIII

Alcoholic extract of beef heart (antigen—2 per cent solid content), cc.	1.6	1.6	1.6	1.6	0.8	0.8	0.4	0.2
Cholesterin (2 per cent solution in alcohol), cc.	0	0.1	0.2	0.4	0.4	0.8	0.8	0.8
Alcohol, cc.	0.4	0.3	0.2	0	0.8	0.4	0.8	1.0
Cholesterin: antigen ratio	0	1/16	1/8	1/4	1/2	1/1	2/1	4/1
Dilution in NaCl N/7. 1 cc. of alcoholic mixture into x cc. total volume; dilutions obtained contain same quantity of antigen	1–80	1–80	1–80	1–80	1–40	1–40	1–20	1–10

In brief, the effect of cholesterin in increasing the sensitiveness of the antigen in the Wassermann reaction is due to the fact that the larger particles formed by cholesterinized antigen when it is diluted

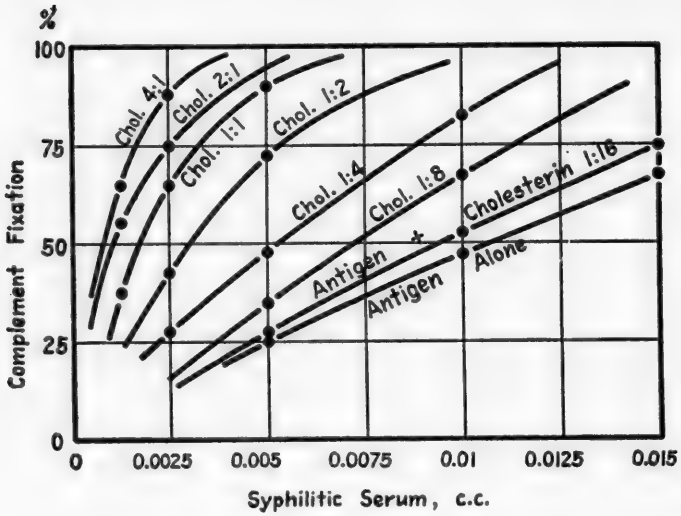

FIG. 3. Effect of cholesterin upon efficiency of antigen in the Wassermann reaction.

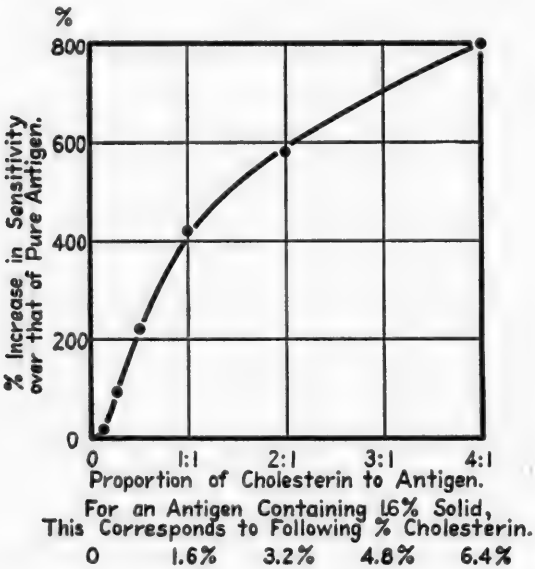

FIG. 4. Effect of cholesterin upon efficiency of antigen in the Wassermann reaction.

with water or saline have a markedly greater avidity for reagin than the submicroscopic lipoid particles formed by antigen alone.

A very significant verification of practical importance is found in the following experiment. The amount of cholesterin used to sensitize the antigen for the Wassermann reaction varies anywhere from 0.05 to 0.2 per cent. The per cent of solids in the antigen is also variable, ranging from 1 to 2 per cent, a maximum cholesterin antigen ratio of 1/5 and a minimum of 1/100. As already shown, the effect of cholesterin upon the particle size in an antigen sol does not stop at this ratio, but continues indefinitely, the only limiting factor being its solubility in alcohol.

If our thesis is correct, *i.e.*, if the sensitizing effect of cholesterin upon Wassermann antigen is due solely to its effect upon the particle size, this effect should not stop at the commonly employed cholesterin: antigen ratio of 1/5, but should increase indefinitely so long as there is any observable effect upon particle number and size (Protocol 5 and Figs. 3 and 4).

Protocol 5

A series of antigen dilutions in NaCl N/7 were prepared as outlined in Table VIII, each containing the same amount of antigen, but increasing quantities of cholesterin.

The minimum quantity of a strongly positive syphilitic serum which would give 75 per cent complement fixation (a 4+ reaction) was determined with 0.4 cc. of each antigen dilution and 0.4 cc. of 1:10 complement by a method described in detail elsewhere (Eagle, 1930, 1). The experimental data are summarized in Figs. 3 and 4.

As predicted, the fortifying action of cholesterin continues indefinitely. An increase is still effective when the cholesterin antigen ratio is already 2/1, *i.e.*, some 10 times as much as is used routinely. For an antigen containing 1.5 per cent solid, this means that the addition of cholesterin up to 3 per cent does not exhaust the possibilities of sensitization. The obvious implication is that cholesterin should be added up to the limit of its alcohol solubility: antigen containing 0.5 per cent cholesterin is roughly 1½ times as sensitive as one containing but 0.2 per cent. Even more suggestive is the fact that the discovery of a very alcohol-soluble substance similar to cholesterin would increase

the sensitivity of the Wassermann antigen at least two-fold, for it could be added up to 1, 2 or even 3 per cent concentration (see Section V).

IV. Explanation of the Effect of Cholesterin in the Flocculation Reactions

It has already been shown (Eagle, 1930, 2) that the flocculation of antigen by syphilitic serum is in all respects analogous to bacterial or red cell agglutination, or foreign protein precipitation by an antibody serum. The reagin globulin of syphilitic serum is deposited as a film of denatured protein around the individual lipoid particles, with a much higher critical potential than the underlying lipoid antigen (10 to 15 millivolts instead of 1 to 5), and therefore much more sensitive to the flocculating action of electrolytes. Relatively small quantities of NaCl (N/20) suffice to depress the protein-coated particles below this critical value, whereupon they can cohere. The diagnostic criterion is the formation of either optically visible aggregates (Kahn, Sachs Georgi) or of a single cohesive floccule (Meinicke Klarungs reaction, Hinton agglutination test, Müller Ballungs-reaction, Lentochol reaction, etc.).

The coarsened dispersion state of the antigen already shown to be caused by cholesterin completely explains its effect in increasing the sensitivity, *i.e.*, the velocity of this aggregation.

1. The first and probably most important factor is the increased avidity of large particles of antigen for reagin, as already discussed in connection with the Wassermann reaction (Section III).

2. *Effect of Cholesterin Bulk.*—The individual particles of the antigen-cholesterol sol are, to all intents and purposes, pure antigen. The cholesterin core, masked by the antigen surface, acts as an inert filler, replacing just so much antigen. Adding cholesterin 0.5 per cent to an antigen already containing 1½ per cent solid is equivalent to increasing the antigen content to 2 per cent. This would result in a correspondingly more rapid flocculation (Eagle, 1930, 4), partially explaining this sensitizing action of cholesterol.

3. *Effect of the Size of Particles upon the Velocity of Their Aggregation.*—It is obvious that the more finely divided a given quantity of

antigen, the more difficult is the formation of optically visible aggregates. In other words, a coarser dispersion would correspond to a preliminary quasi-aggregation, facilitating by just so much the subsequent aggregation by syphilitic serum. Moreover, with coarser particles there is less surface, and more reagin per unit surface, which also increases the velocity of aggregation (Eagle, 1930, 4).

Qualitatively, therefore, cholesterin would accelerate flocculation because it causes a coarser dispersion of antigen. The quantitative evaluation of this effect must await the ultramicroscopic measurement of the particle sizes in an antigen, as contrasted with an antigen-cholesterin, sol. Thus, if the average particle in an antigen sol measures, *e.g.*, 0.05μ radius, while the average particle formed by a cholesterinized antigen measures 0.5μ, an optically visible aggregate of, say, 2μ radius would necessitate the cohesion of 6×10^4 particles of the one, and only 64 of the other.

The foregoing applies with even greater force to those reactions in which the diagnostic criterion is not just visible aggregation, but sedimentation (Müller, Lentochol, etc.). The velocity of sedimentation is proportional to the square of the radius of the aggregate: and the larger the initial particles the fewer need aggregate in order to produce rapid sedimentation.

To summarize, the effect of cholesterin in increasing the sensitivity of antigen in the flocculation tests is due to many factors, the relative importance of which it is difficult to evaluate. They are listed below in what is considered to be the order of increasing importance.

1. Cholesterin acts as just so much added antigen, known to increase the sensitivity of flocculation.

2. With fewer and larger particles, there is less surface per unit antigen and therefore more reagin per unit surface, with a corresponding increase in the velocity of flocculation.

3. The coarse dispersion of antigen caused by cholesterin facilitates subsequent aggregation. Fewer particles need cohere to form visible or sedimenting clumps.

4. Large particles such as those seen in an antigen-cholesterin sol have an enormously greater avidity for reagin than the small particles formed by antigen alone.

V. SUMMARY AND DISCUSSION

When cholesterinized antigen is dropped into an excess of water, the rapid flocculation of cholesterin crystals is prevented by the fact that, as tiny aggregates form, they adsorb a protective surface of hydrophilic lecithin (*i.e.*, antigen) which endows the particles with its own stable surface properties and thus prevents further aggregation. The colloidally dispersed antigen-cholesterin particles have approximately the same isoelectric point (pH 1.9), critical potential (1 to 5 millivolts) and coagulation value (0.75 M NaCl) as pure antigen particles of the same concentration, while the corresponding values for cholesterin are pH 2.1 to 3.4 (probably due to an associated impurity), >100 millivolts, and <0.001 N NaCl, respectively.

Presumably, this adsorption of antigen by the cholesterin nucleus is determined by the fact that the former has a lower surface tension against water. At any rate, many surface active substances (serum; alcoholic extract of milk, egg or any animal tissue; Na-oleate; Na-glycocholeate; Na-taurocholate) cause a similar stable dispersion of cholesterin; and conversely, many otherwise water-insoluble substances of the most diverse chemical structure can be made to form a stable colloidal suspension by adding antigen to their alcoholic solutions before dropping into water.

The colloidal suspension formed by antigen alone is very finely dispersed: only a few of the particles exceed the limits of dark field visibility. Cholesterin causes a marked increase in the number of these particles, out of all proportion to its mass; thus, one part of cholesterin to five of antigen causes a ten-fold increase in such visible particles, at the expense of the submicroscopic micellae formed by antigen alone. At the same time, the suspension becomes much more turbid. The particles remain discrete until the cholesterin: antigen ratio exceeds 1:1, when slight microscopic aggregation is observed; microscopic flocculation is seen only when this ratio exceeds 5:1, when there is not sufficient antigen to act as an efficient protective colloid.

Cholesterin therefore causes a coarsened dispersion of antigen by forming a relatively large nucleus upon which antigen is adsorbed. As shown in the text, the larger the antigen particle the greater is its avidity for reagin per unit surface or mass. Thus, the coarse sol formed

by dropping water-into-antigen is about twice as efficient as a finely dispersed antigen-into-water sol of the same concentration. The coarsened dispersion caused by cholesterin completely explains the greater sensitivity of the cholesterinized antigen in complement fixation.

The same factor obtains in the flocculation reactions. In addition, the coarsened dispersion acts as a preliminary quasi-aggregation, facilitating by just so much the subsequent formation of visible clumps (or sedimenting aggregates) upon the addition of syphilitic serum; moreover, there is less surface in a coarse sol, with more reagin per unit surface, and correspondingly more efficient flocculation.

The foregoing would be of purely academic interest were it not for the following considerations. From several points of view cholesterin is unsatisfactory as a sensitizer for antigen. Its solubility in alcohol is small. Even the 0.6 per cent concentration used in the Kahn test is difficult to keep in solution. Yet, as our experiments show, its sensitizing action increases indefinitely with its concentration. If it were sufficiently soluble, even 3 per cent could be used to advantage, increasing the sensitivity of 1½ per cent antigen for complement fixation some 200 to 400 per cent, instead of about 50 per cent, as does 0.2 per cent cholesterin.

Since, as we have shown, the sensitizing action of cholesterin upon antigen is due solely to the coarse dispersion it causes, and since it is quite inert during the actual combination of the lipoid particles with reagin, it can be replaced by any substance with similar physical properties. The problem in hand was therefore to find a water-insoluble substance, very soluble in alcohol, with so high an interfacial tension against water that, as in the case of cholesterin, microscopic particles would adsorb antigen when the alcoholic solution of the two is dropped into water. Given such a substance, it would be possible to obtain a more sensitive antigen for both complement fixation and flocculation, but particularly for the former.

These theoretical expectations have been realized in a group of substances shortly to be reported: they make possible an antigen which is from 2 to 10 times as efficient in the Wassermann test as any now available.

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(2) *J. Exp. Med.*, 1930, **52**, 717.
(3) *J. Exp. Med.*, 1930, **52**, 739.
(4) In preparation.
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THE KILLING OF COLON BACILLI BY X-RAYS OF DIFFERENT WAVE LENGTHS

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The experiments to be described in this paper are quantitative studies of the rate at which colon bacilli die when subjected to known intensities of X-rays of various wave lengths. Such measurements are of interest for several reasons. They provide a more or less accurate description of the course of this biological reaction and they throw a definite, though as yet an indirect, light upon the mechanism by which these cells are killed. At the same time they provide an essential basis for attempts to alter cells without killing them and they give data which have a direct bearing upon the validity of the generally accepted principles of X-ray dosimetry as applied to tumor and other biological irradiations.

The biological[1] and physical[2] procedures are the same as those previously described. A few single bacilli (*ca.* 200 to 300 per square inch) were spread as evenly as possible upon the surface of an agar plate and two equal areas were stamped upon it. One of these areas was immediately given a measured dose of X-rays, the other served as a standard. The ratio of the counts made after incubation of the number of colonies growing out in the irradiated and standard regions is the survival ratio. In any one experiment the X-ray intensity was held constant and exposures of enough different lengths of time were made to determine the course of the survival ratio-dosage curve. In order that a sufficient number of bacteria should be counted to make the results statistically significant, at least ten plates were irradiated for each of the chosen exposure times and their results averaged. In this way each final survival ratio is the result of counts of not less than 2000 to 3000 organisms. Though the quantities thus obtained are called survival ratios,

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[1] Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, **51**, 921.

[2] Wyckoff, R. W. G., *J. Exp. Med.*, 1930, **52**, 435.

they are in reality measures of the destruction of the reproductive function in the bacteria. It is possible, particularly with the softer radiations, that this loss of ability to divide takes place without cell death.

The previously described[3] gas tube, equipped with targets of different metals, has been used to obtain the intense beams of X-rays needed for these experiments. In this way, killing rates upon *B. coli* have been measured for X-ray beams consisting mainly of the K lines of silver, molybdenum, copper and chromium and the L lines of silver. Because of the loss of intensity consequent upon filtration and the desirability of having the data with the different radiations as comparable as possible, no attempt has been made to render the beams in any of these experiments more nearly monochromatic. The earlier experiments with filtered and unfiltered copper rays indicate, however, that only a moderate error is introduced by this heterogeneity.

The current through the X-ray tube during the irradiations was held constant at either 4 or 8 milliamperes. The peak voltage across it depended on the target —for Ag K, Cu K and Cr K rays it was 34 KV, for Mo K it was 34 or 38 KV and for Ag L, 21 KV as measured with a 12.5 cm. sphere gap. The beam intensity was determined using the small air ionization chamber described in the earlier experiments with copper rays. The molybdenum and silver K radiations are sufficiently hard so that this air ionization was taken as a direct measure of the X-rays striking the irradiated agar surface. With the other and softer rays, correction was made for the amount absorbed in the layer of air between the surface of irradiation and the volume giving the ionization current. The length of this air column was 3.2 cm. Taking the absorption coefficient[4] of Cu K radiation as $\mu/\rho = 8.43$, of Cr K as 28.0 and Ag L ($\lambda = 3.98$ A) as 129.3, calculation shows that the measured ionizations are 97 per cent for Cu K, 89 per cent for Cr K and 59 per cent for Ag L of those due to the X-rays at the irradiated surface. Introducing this absorption correction and transferring to standard air conditions, the measured air ionizations of the beams used in the experiments of Table I are recorded in this same table as the saturation currents due to the ions produced in a cube 1 cm. on its edge.

Typical experiments giving survival ratios with each of the foregoing radiations are reproduced in Table I. The ratios thus obtained, as well as those from several similar experiments, are plotted on a semilogarithmic scale in Figs. 1 to 5. It is evident that the results with all radiations lie on straight lines in these diagrams.

[3] Wyckoff, R. W. G., and Lagsdin, J. B., *Radiology*, 1930, **15**, 420.

[4] Kaye, G. W. C., X-rays, London, 1923, 4th ed., 145; Jönsson, E., Uppsala Univers. Arsskrift, 1928.

TABLE I

Survival Ratios for Various Radiations

Irradiation times	Survival ratios for				
	Ag K (0.564A)	Mo K (0.710A)	Cu K (1.537A)	Cr K (2.29A)	Ag L (3.98A)
seconds					
5	—	—	0.714	—	—
10	—	0.863	0.687	0.629	—
20	0.676	0.693	0.512	0.392	0.689
30	—	0.612	0.277	0.293	—
40	0.443	0.491	0.220	0.185	0.518
50	—	—	—	0.128	—
60	0.334	0.380	0.149	0.100	0.322
80	—	0.300	—	—	—
90	0.192	—	—	—	0.263
120	0.124	—	—	—	0.173
Ionization per sec./cm.^{2} in 1 cm. of air	73.1	94.1	173.1	274.5	133.8 e.s.u.

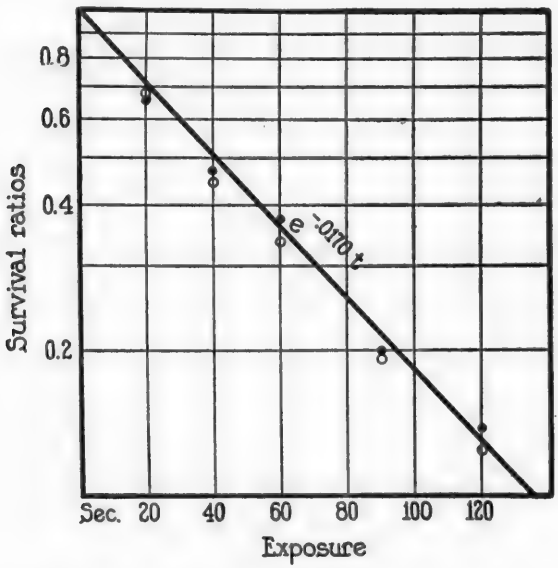

FIG. 1. Data from two sets of experiments with a tube giving silver K radiation. The ratios of Table I are plotted as the open circles.

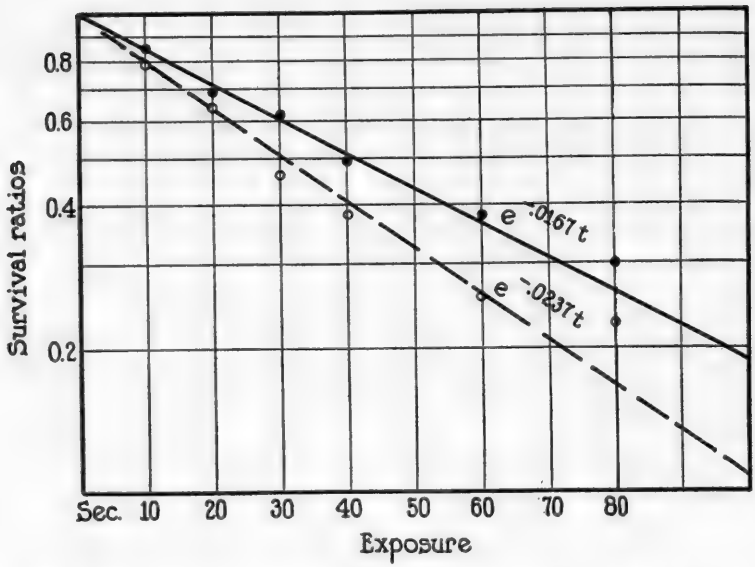

FIG. 2. Data from two different sets of experiments with a tube giving molybdenum K radiation. The ratios of Table I are plotted as full circles.

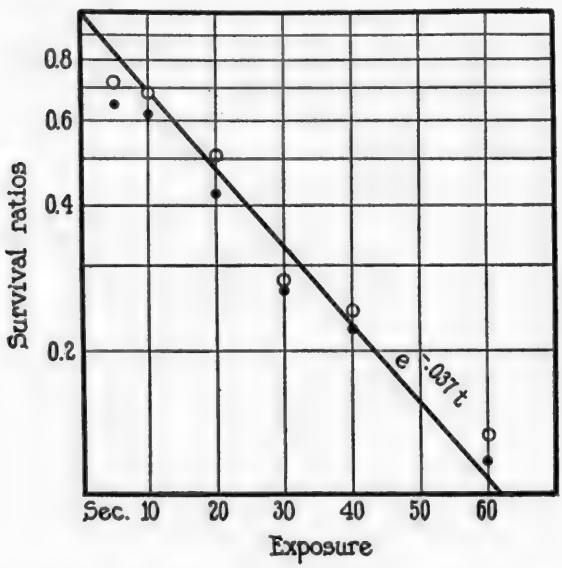

FIG. 3. Data from two sets of experiments with a tube giving copper K radiation. The ratios of Table I are plotted as open circles.

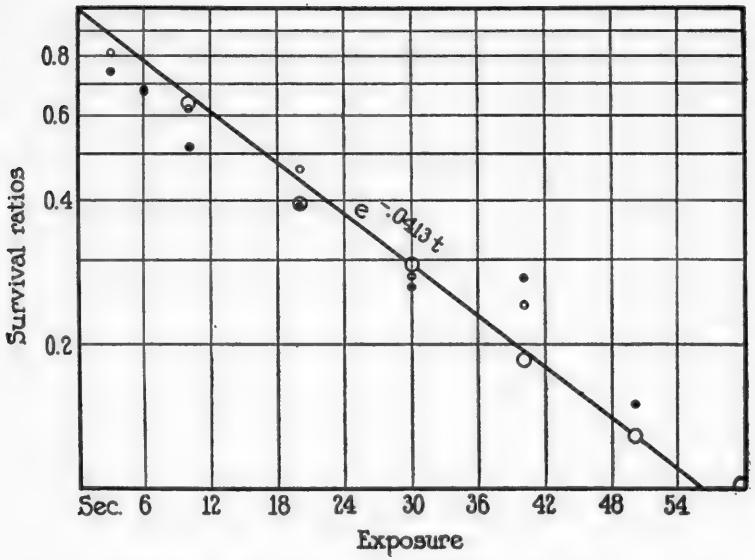

FIG. 4. Data from three sets of experiments with a tube giving chromium K radiation. The ratios of Table I are plotted as the large open circles.

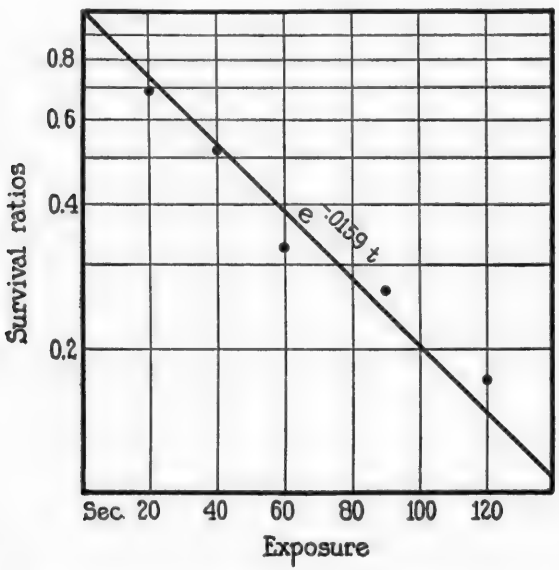

FIG. 5. Data from the set of experiments with a tube giving silver L radiation that is recorded in Table I.

Analysis

The same analysis based upon the quantized absorption of X-rays which was outlined previously for copper rays is applicable to all these experiments. From this standpoint the straight lines of the figures are proof that the absorption of one quantum of X-ray energy is sufficient to produce death even with the very soft silver L radiation. This agrees with the results of Holweck[5] and Lacassagne[5] with *B. pyocyaneus*. As before, the average or expected number of absorptions per bacterium can be computed for each experiment from the beam intensity, the size of the bacterium and a knowledge of the number of ions liberated in air by one quantum of the wave length used. A typical calculation[6] showing the quantities employed throughout has already been outlined. The expected numbers of absorptions, computed for the several wave lengths of this series of experiments, are listed as "Calculated α" in Table II. Since the survival ratios give straight lines when plotted semilogarithmically, they must be expressed by an equation of the type

$$A_1/A_0 = \text{survival ratio} = e^{-\alpha' I} \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

The α''s that are given by the slopes of the lines of Figs. 1 to 5 are listed in Table II. If every quantum absorbed were deadly, these α''s would equal the calculated values of α. Since they are much smaller, their ratio, α/α', furnishes the average number of absorptions which occur before a bacterium is killed. If the volume through which a quantum acts were small compared to that of the vital parts of a cell, then the reciprocal of this quantity, or α'/α, would measure eht volume of the vital elements. Physical experiments indicate that this is not the case, however, and as a consequence the ratios α'/α, at least for the harder X-rays, are more an index of the size of the quantum's sphere of action than of the bacterium's vital elements.[7]

[5] Holweck, F., *Compt. rend.*, 1929, **188**, 197; Lacassagne, A., *Compt. rend.*, 1929, **188**, 200.

[6] Wyckoff, R. W. G., *J. Exp. Med.*, 1930, **52**, 435.

[7] It is interesting to note in this connection that the curve showing the increase in sensitive volume with harder rays is very similar to curves that are obtained when the range (in air) of electrons is plotted against their speed (*cf.* the data of Lenard and of Wilson quoted by H. Küstner in Ergebnisse d. med. Strahlen-

Nevertheless, it can with safety be concluded that these cell constituents which are essential to its life, or at least to its growth through multiplication, cannot in their sum total have a greater relative volume than α'/α. The "sensitive volumes," α'/α, furnished in this way by the several radiations studied are given in Table II. Together with their reciprocals they are shown graphically in Fig. 6. Since α'/α can be as small as 0.014 (for Ag L radiation), the region within a colon bacillus which must be injured to prevent cell multiplication cannot be more than this fraction of the bacterial volume. The manner in which the curve of Fig. 6 flattens out with increasing wave length suggests that the true sensitive volume of this organism may not be far from 0.01. These experiments cannot show whether

TABLE II

	Ag K	Mo K	Cu K	Cr K	Ag L
Calc. α	0.079	0.126*	0.527	1.220	1.108
Obs. α'	0.0174	0.0167*	0.0370	0.0413	0.0159
Sensitive volume $\frac{\alpha'}{\alpha}$	0.220	0.159	0.0702	0.0338	0.0144
Hits to kill $\frac{\alpha}{\alpha'}$	4.54	6.46	14.2	29.5	69.7
β in expression (2)	0.0_3238	0.0_3215	0.0_3231	0.0_3150	0.0_3119

* These numbers refer to the experiment of Table I; the remaining results for molybdenum are averages of more than one experiment.

the sensitive part is a single structure or is the sum of a number of smaller vital elements. If, however, as appears natural, it is associated with the chromatin material of the cell, then the latter is probably true.

The sensitive volume as calculated above possesses a large possible error. At least two factors contribute to this potential inaccuracy. One is the non-monochromatic character of the X-ray beams used, the other and more important lies in the serious physical problems met in measuring such an intense beam of soft X-rays. Consistent readings of intensity have been obtained but extended research is nec-

forschung, Leipzig, 1925, Band I, 229. This suggests that the biological sphere of action of an absorbed quantum is less than the spherical volume within which it liberates all its ions.

essary before their absolute accuracy is satisfactorily known. The previously published results with Cu K rays are in moderate agreement with those of this paper. Most of the difference which exists is, how-

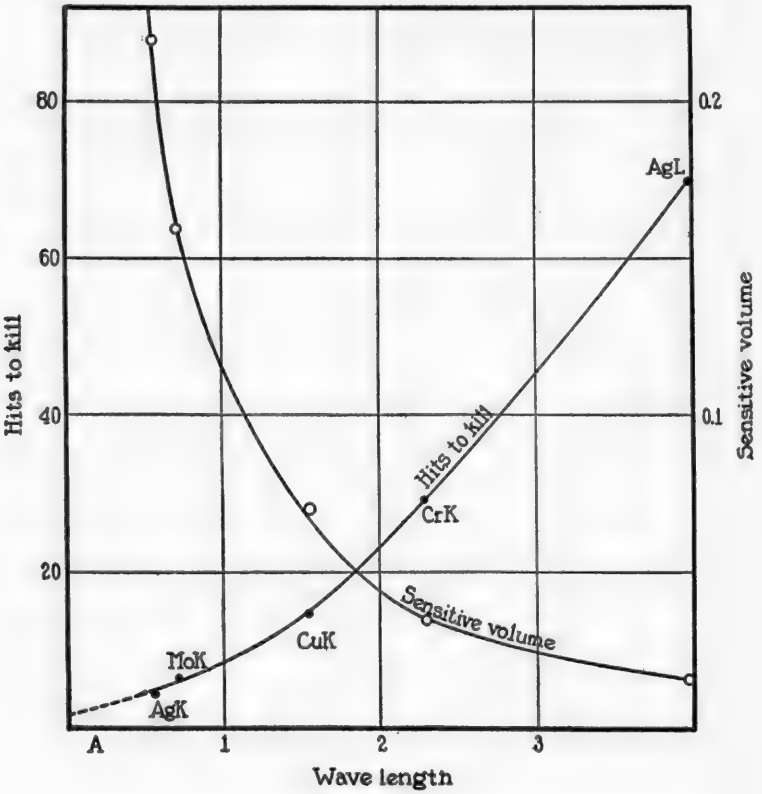

FIG. 6

ever, to be ascribed to improvements which have been made in the measurement of beam ionization.

It is also instructive to state the killing action of these different wave lengths in terms of the energy rather than the quanta absorbed. This

can readily be done through the measured air ionization using an equation of the form

$$A_1/A_0 = e^{-\beta u} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

where u is expressed in electrostatic units per second per cubic centimeter of air under standard conditions (in roentgen units). The exponents β as found for the wave lengths used are given in Table II. If the killing were proportional to the number of ions produced (in air and undoubtedly also in the bacteria) these β's should be constant. For the very soft Cr K and Ag L radiations they are definitely smaller than for shorter X-rays. If the same amount of energy is consumed in producing an ion pair in air for all these radiations, as the best available information[8] indicates to be true, then this result must mean that more energy is needed to kill a bacterium when the quantum is small.

DISCUSSION

Throughout the analysis which has been followed in interpreting these experiments, it has been considered that differences which might exist in the resistances of individual organisms to X- and cathode rays were not important. With the high velocity electrons of a cathode ray beam where nearly every electron that is absorbed brings about death, this conclusion is a necessary one. It is a strong argument in favor of its continued applicability that the killing rate of bacteria is the same for these electrons and for X-rays throughout the range of wave lengths studied.

The statement is commonly encountered that whenever bacteria are subjected to an unfavorable environment, be it physical or chemical, they die off at a rate which is logarithmic for at least part of the time. By many it has been thought that such a decrease in bacterial count meant that death of these cells was a monomolecular chemical reaction; others have asserted that these curves of death are indices, not of the mechanism of killing, but rather of the relative sensitivities of the bacteria in the cultures used. The comparative merits of these two points of view have been discussed frequently but not conclu-

[8] Kulenkampff, H., *Ann. d. Physik*, 1926, **79**, 97.

sively.[9] If relative sensitivities were the predominant factor, then it might be expected that the death-rate curves for a particular organism would be much the same irrespective of the killing agent. In making such comparisons the entire curve and not merely its middle portion is significant. When this is taken into account the curves for the action of different physical agents are often found to be of unlike shape and are not necessarily semilogarithmically linear. Thus experiments on the death-rates of spores and of some actively metabolizing bacteria indicate that these rates depend upon both the environment and the type of organism. The curves of some of these rates[10] seem semilogarithmically linear; others[11] depart widely from such a relation.

Few of the studies of the effects of radiations on cells give useful data. The death-rate of staphylococci when subjected to ultraviolet light of a single wave length has been measured.[12] These results, plotted on a suitable semilogarithmic scale (Fig. 7), give a curve which is not as straight as the line obtained when this bacterium is irradiated with cathode rays but is nevertheless of a smooth multiple-hit type. Lacassagne and Holweck have found that the death-curves of *B. pyocyaneus* are straight if killing is effected with Ag L rays but not with the still softer Al K radiation. These facts are opposed to the idea that the killing-curves with which they are concerned are primarily expressions of relative sensitivity.

Though it is quite clear that this factor of "biological variation" cannot be of predominant importance in either the X-ray experiments with *B. pyocyaneus* or in these observations on *B. coli*, no conclusive data exist to prove that it does not occur as a secondary factor to influence the foregoing analysis. The experiments bearing most directly on this problem are the recent observations of Gates[13] showing that except possibly in strongly alkaline solutions the survival ratios of *S. aureus* irradiated by ultraviolet light are independent of the pH of the medium in which they grow. Since death-curves with heat are known

[9] See for example papers mentioned in Rahn, O., *J. Gen. Physiol.*, 1929, **13**, 179.

[10] Bigelow, W. D., *J. Inf. Dis.*, 1921, **29**, 528.

[11] See the chart reproduced by Esty, J. R., in Jordan, E. O., and Falk, I. S., The New Knowledge of Bacteriology and Immunology, Chicago, 1928, 299.

[12] Gates, F. L., *J. Gen. Physiol.*, 1929, **13**, 231.

[13] Gates, F. L., *J. Gen. Physiol.*, 1929, **13**, 249.

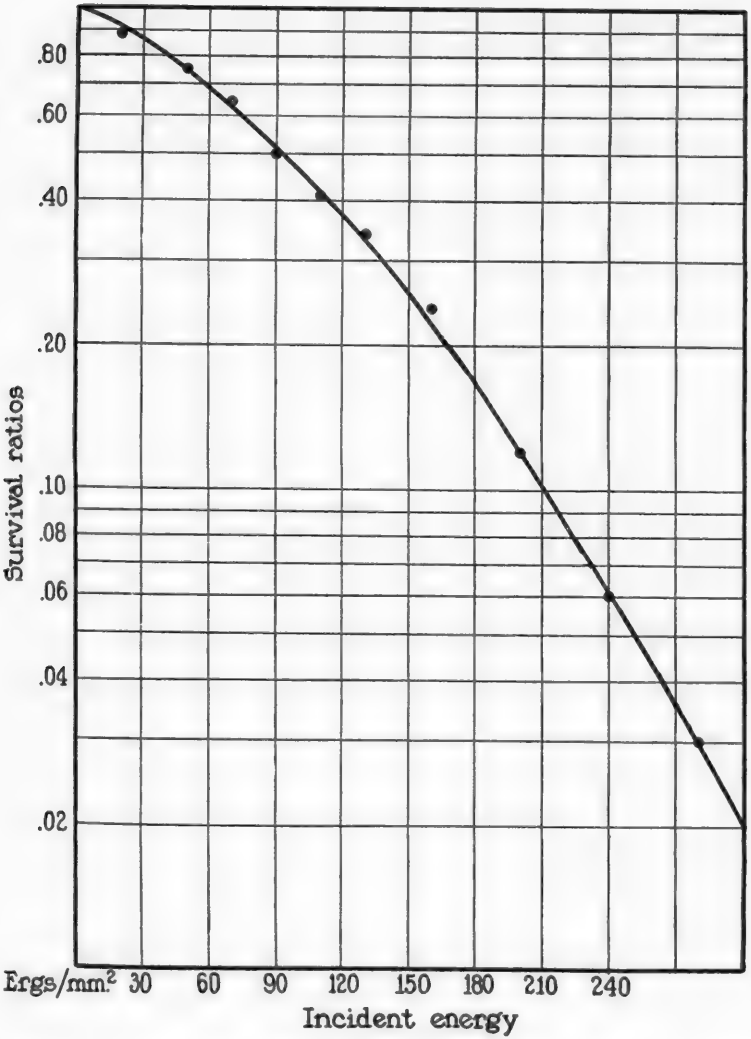

FIG. 7. A plot of the data of Gates showing the killing rates of *Staph. aureus* for light of wave length $\lambda = 2540$ A.

to be influenced by the acidity of the medium,[14] this is strong evidence that killing rates with ultraviolet radiation are not concerned with the sensitivity of the organisms as measured by some other physical agents. It is to be expected that sensitivity should be even less important with X-rays.

In so far as results with a relatively small bacterial cell are applicable to large tissue cells, the measurements of the amount of ionization necessary to produce death for different radiations have a bearing on the principles of X-ray dosage. The general constancy of β for the harder rays of Table II is in good agreement with the assumption that ionization, as determined by an air chamber, is an accurate measure of the biological action of the rays. The smaller β's found for very soft rays are, on the other hand, an indication that air ionization data with a Bucky "grenz-ray"tube, for example, are probably not, from a biological standpoint, accurately comparable with those from the more ordinary types operated at higher voltages.

Valuable help in carrying out these experiments has been given by Charles G. Porskieves.

CONCLUSIONS

X-ray beams of wave lengths lying in the range between 4 A and 0.5 A all kill *B. coli* in a semilogarithmically linear fashion. Interpreted in terms of the known quantized absorption of X-rays, this means that one absorption of any of these radiations is sufficient to kill. Though death results from a single absorption, only about one hit in four with Ag K and one in sixty with Ag L radiation is deadly. The course of curves constructed from these experimental results suggests that the portion of this bacterium which is essential to its continued life has a total of approximately 0.01 of the cell volume.

For copper and harder radiations the biological action of the rays is proportional to their measured air ionization. The same biological change with the softer chromium K and silver L X-rays seems to require a somewhat more intensely ionizing beam.

[14] Bigelow, W. D., *op. cit.*

THE EFFECT OF BACTERIAL VARIATION UPON THE FACTORS NECESSARY FOR THE PHENOMENON OF LOCAL SKIN REACTIVITY TO BACTERIAL FILTRATES

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If an injection of a bacterial filtrate into the skin of a rabbit is followed 24 hours later by an intravenous injection of a culture filtrate of the same or another bacterium there appears an extremely severe hemorrhagic necrosis at the prepared skin sites. This phenomenon of local skin reactivity was described by the present author (1–12) and corroborated by Hanger (13), Ecker and Welch (14), and Frisch (15). Among the various phases of the phenomenon thus far studied there has been reported the specific neutralizability of the reacting factors (*i.e.*, intravenous factors) by homologous immune sera (9, 10). The object of the work presented in this paper was to study the serum neutralizability of reacting factors derived from various stock strains of the same bacterium and also to determine the influence of bacterial variation upon the serum neutralizability of these factors.

EXPERIMENTAL

Horse and rabbit immune sera were employed in this work. The immunization of rabbits was carried out by semi-weekly simultaneous subcutaneous and intravenous injections. Filtrates of 6 day old cultures in tryptic digest broth were injected subcutaneously. Unwashed heat-killed vaccines were injected intravenously. The doses were increased by 25 per cent at each injection. The injections were carried out for 4 to 6 weeks. 25 cc. of the filtrate was the largest subcutaneous dose and 20 cc. of the vaccine (1 billion organisms per cubic centimeter) was the largest intravenous dose. In attempting to prepare sera against *B. typhosus* rough variants and against a strain of *B. typhosus* passed through mice (Ty 159 Mouse $_{46}$), considerable difficulties were encountered because of the high mortality rate of the rabbits under treatment. The procedure of immunization was then modified for these strains, as follows:

The initial dose of vaccine was 100,000,000 organisms. The largest dose given

at the last injection was 5 billion organisms. Instead of filtrates of tryptic digest broth cultures, filtered washings of 24 hour old cultures on solid media were used for subcutaneous injections. The first dose was 0.1 cc. The largest dose was 3 cc.

Horse sera were prepared according to the method described in a previous publication (9). The length of time during which the immunization was continued before each bleeding is indicated in Table II.

The *B. typhosus* strains used for the experiments about to be described were designated Ty T_L stock, Ty 157 stock, Ty 159 stock, Ty 864 stock, Ty 870 stock and Ty 240 stock. The *B. coli* strains also employed for some experiments were called C 42 and C stock. These strains were kept in the laboratory collection for from 1 to 4 years prior to this work.

The colony appearance of these strains on plain agar, with the exception of Ty T_L stock and C 42 stock, was of the normal smooth type. The Ty T_L stock strain showed colonies which appeared distinctly granular under the low dry power. Their margin was ameboid and somewhat ragged but this was not sufficiently pronounced to classify this strain as rough. This intermediate type of roughness was, however, a stable characteristic which persisted during the entire year of observation without any change either in the direction of smoothness or more pronounced roughness. This stability was ascertained by 42 "platings" of this strain. No typical rough colonies were obtained from old fluid cultures.

The *B. coli* strain C 42 stock, kindly sent to me by Dr. André Gratia 6 years ago, was a typical rough strain obtained by him from a bacteriophage lysed culture. The colonies were cauliflower and coarsely granular.

Broth cultures of the various strains, with the exception of Ty T_L stock and C 42 stock strains, gave uniform growth after 24 hours of incubation at 37.5°C. The Ty T_L stock strain produced, in addition to the uniform cloudiness, an insignificant amount of precipitate which on shaking gave rise to easily broken up flakes. The C 42 stock strain grew only on the bottom of the test tube, the supernatant broth remaining perfectly clear.

All the *B. typhosus* stock strains showed well pronounced motility.

24 hour cultures of various strains on plain agar were washed in distilled water by centrifugalization and resuspended in 0.85 per cent and 1.4 per cent NaCl solution. The suspensions were incubated in a water bath at 37.5°C. for 2 hours and then placed in the refrigerator for 22 hours. The C 42 stock suspension was completely agglutinated but the remaining strains stayed in suspension.

The various strains were grown in immune sera in order to obtain rough variants. The results were as follows:

12/2/1929, the Ty T_L stock strain was inoculated into plain broth containing 1–10 dilution of Serum R-238 (homologous antityphoid rabbit serum). After 24 hours of incubation at 37.5°C. the growth appeared clumped. The culture was further incubated at room temperature until 12/17/1929. At this date it was plated out on plain agar. The colonies appeared typically rough. 12/18/1929, a rough colony was fished into broth containing 1–10 dilution of Serum R238 and

incubated at 37.5°C. for 24 hours and later at room temperature. 1/17/1930, the culture was plated out on plain agar and a single colony which appeared distinctly rough was transplanted onto a plain agar slant. The rough strain thus derived from the Ty T_L stock strain was designated Ty T_L 238 ser_2 strain. This strain grew in plain broth in flaky clumps leaving the supernatant broth perfectly clear; it gave complete agglutination in 0.85 per cent and 1.4 per cent NaCl solutions and was non-motile. The fermentation of sugars was typical of *B. typhosus*. The Ty T_L 245 ser_2 strain was prepared from the Ty T_L stock strain in an identical manner except that Serum R245 was used (homologous antityphoid rabbit serum). The strain was also typically rough as ascertained by the appearance and above described tests. Antityphoid bacteriophage, obtained from a convalescent typhoid patient in the usual manner, was also employed in order to induce bacterial dissociation, as follows:

1/15/1930, a tube of broth containing 1–10 dilution of the bacteriophage was inoculated with a young culture of Ty 870 stock strain, incubated for 24 hours at 37.5°C. and at room temperature for 48 hours and then seeded onto a plain agar plate. The colonies which appeared rough were fished into broth containing 1–10 dilution of the same bacteriophage and again incubated as above until 1/22/1930. At this date a third passage of a rough colony was made through the bacteriophage. The culture plated out (1/29/1930) gave a number of distinctly rough colonies. A culture on plain agar derived from a single colony was designated Ty 870 $phage_3$. The strain persistently retained its roughness as ascertained several times by the tests in plain broth, NaCl solutions, and unchanged appearance of growth on solid media.

B. coli C stock strain was converted into a rough strain by means of seven daily passages through 10 per cent Serum H_{148} (anti-*coli* horse serum) broth. The 8th passage yielded typically rough colonies, as proven by the above described tests. This strain was designated C rough.

The other strains of *B. typhosus* employed here did not undergo any dissociation in spite of persistent attempts, as shown in the following protocols:

Ty 864 stock was passed 12 times through 1–10 dilution of antityphoid horse serum in plain broth. The culture of each passage was incubated for 24 hours at 37.5°C. and 9 days at room temperature, and plated out on plain agar at the end of this period. A single colony appearing rough was used for the next passage. Four passages yielded colonies which appeared questionably rough. The colonies, however, promptly reverted to the normal appearance when subcultured on plain agar for several generations. The same strain similarly passed through the antityphoid bacteriophage did not undergo any change. The other two strains of *B. typhosus* employed in this work (*i.e.*, Ty 159 and 240 stock) were also passed a number of times through antityphoid immune horse and rabbit sera and antityphoid bacteriophage. No dissociation was accomplished in spite of a large number of passages.

The various *B. typhosus* stock strains were also passed through mice in order

to increase the virulence of the strains and possibly enhance their smoothness. The technique adopted for the purpose was, as follows:

A 24 hour old agar slant of a given strain was emulsified in 0.85 per cent NaCl solution to a turbidity representing approximately 1 billion organisms per cubic centimeter. 1 cc. of the emulsion was injected intraperitoneally into 2 mice. 24 hours later cultures were made on Endo agar plates from the heart's blood of the dead or killed mice. The 24 hour old growth on the plates was suspended to the above indicated turbidity in 0.85 per cent NaCl solution. One half of 1 cc. of the emulsion was used for injection into a mouse. Only heart's blood cultures were employed for passages. The strains passed through mice were designated mouse strains, with the name of the stock strain from which they were derived and a number indicating the number of passages. None of the stock strains (including the Ty T_L strain) underwent any change in the appearance of the colonies in spite of numerous passages.

The different stock strains of *B. typhosus* and *B. coli*, the variants obtained by means of immune sera and bacteriophage and the strains passed through mice were utilized as follows:

I. Serum Neutralizability of B. typhosus and B. coli Reacting Factors

a. Preparation of Toxic Filtrates.—The toxic filtrates necessary for the phenomenon of local skin reactivity were "agar washings" filtrates prepared in a manner previously described (6, 7). These filtrates were designated by their number and the name of the strain employed for their preparation. The "mouse" strains did not undergo more than one passage through artificial media previous to their use for the preparation of toxic filtrates. The 24 hour old heart's blood cultures on Endo agar plates were seeded into plain broth. The plain broth properly diluted served as the inoculum for the Kolle flask agar cultures necessary for the preparation of the filtrates.

b. Titration of the Reacting Factors.—The quantitative measurement of the reacting factors was carried out as follows:

The rabbits used for titrations were each injected intradermally with 0.25 cc. of the undiluted filtrate and divided into groups of four. 24 hours later a single intravenous injection of the filtrate diluted in 0.85 per cent NaCl solution was given to each rabbit. The dose was 1 cc. per kilo of body weight. Each group of rabbits received intravenously a different dilution of the filtrate. The local reactions were read 4 to 5 hours after the intravenous injections. The titrations were carried until the lowest dilution was found which gave no reaction in the 4 rabbits tested as well as the highest dilution which gave reactions in one or more rabbits of the group. The minimal dose of reacting factors was then considered as lying between these two figures. If a given filtrate was employed for any length of time, repeated control titrations were done. In these control tests, the dilutions employed were both the highest dilution capable of eliciting reactions and the lowest dilution giving no reactions. This was necessary since a loss of

potency was obtained during storage of filtrates (10, 11) as well as an occasional increase in the potency such as was recently observed.

The above described titration of the reacting factors is different from the method of titration previously described (9, 10, 12). In the older experiments the object was to demonstrate the serum neutralizability of the reacting factors and also to compare the neutralizing potency of various sera against one given batch of filtrate used throughout the work. For this purpose it was sufficient to determine the smallest amount of the filtrate capable of eliciting reactions in a high percentage

TABLE I

The Titer of Reacting Factors in Various Bacterial Filtrates

No. of filtrate	Strain used for preparation of filtrate	Highest dilution of filtrate eliciting reactions	Per cent positive rabbits with highest dilution eliciting reactions	Lowest dilution of filtrate showing no reactions	No. of reacting units per cc. of filtrate	Per cent of positive rabbits with various numbers of reacting units	
						6 reacting units	10 reacting units
A_{30}	Ty T_L stock	1:400	50	1:600	500	—	—
A_{26}	Ty T_L stock	1:800	50	1:1000	900	—	—
A_{36}	Ty T_L stock	1:600	50	1:800	700	80	80
823	Ty T_L 238 ser_2	1:600	50	1:800	700	—	—
778	Ty T_L 238 ser_3	1:700	25	1:800	750	—	—
834	Ty T_L 245 ser_3	1:600	50	1:700	650	—	—
989	Ty T_L 245 ser_2	1:200	50	1:400	300	90	100
852	Ty 157 stock	1:1000	25	1:1200	1100	70	100
846	Ty 864 stock	1:400	25	1:600	500	—	—
848	Ty 159 stock	1:200	50	1:400	300	—	—
779	Ty T_L $Mouse_{42}$	1:300	25	1:400	350	—	—
838	Ty T_L $Mouse_{53}$	1:500	25	1:600	550	—	—
840	Ty 159 Mouse	1:550	25	1:700	625	—	—
971	Ty 159 $Mouse_{46}$	1:1100	50	1:1400	1250	80	80
980	Ty 240 Mouse	1:700	25	1:900	800	—	—
911	Ty 870 $phage_3$	1:700	25	1:900	800	—	—
1042	C stock	1:900	50	1:1100	1000	—	—
985	C rough	1:1000	50	1:1200	1100	—	—

of rabbits. However, in this work, in which studies on the neutralizability of various filtrates derived from different strains were planned, the method of titration as presented in this paper became necessary because of its sharper "end point." Table I embodies the titers of the reacting factors in various filtrates.

As is seen from Table I, all the stock strains tested, as well as their variants were capable of producing highly potent toxic substances.*

* It is interesting to note that provided one area of the skin is prepared the amount of reacting factors necessary to elicit reactions in the skin of certain rab-

As will also be seen from the same table the minimal dose of reacting factors produced reactions only in a small percentage of rabbits in the majority of instances. In planning the neutralization experiments about to be described the question arose as to what was the minimal number of reacting units which would yield a high percentage of reactions and, therefore, could be safely used for these experiments. Various multiples of the minimal doses were tested in groups of ten rabbits. As is seen from Table I, 6 and 10 units already gave a high percentage of reactions.

c. Serum Neutralization of Reacting Factors of Various Filtrates.—The technique employed for the neutralization experiments was similar to one described before (9, 10). One area of the skin of the abdominal wall of a rabbit was injected with 0.25 cc. of an undiluted filtrate. 22 to 24 hours later a single intravenous injection was given of a mixture of the same filtrate (diluted previously in 0.85 per cent NaCl solution to the desired degree) with a given undiluted serum in proportion 4:1. The mixtures prepared on the morning of the experiments were incubated in a water bath at 37.5°C. for 1 hour. The precipitate in the mixtures was broken up by shaking immediately before the injection. The intravenous dose of the mixture was 1.25 cc. per kilo of body weight. The readings of the reactions were made 4 to 5 hours after the intravenous injection. Each mixture was tested in 4 rabbits. If no reactions were obtained in the 4 rabbits tested, the result was considered as showing "consistent" neutralization of the reacting factors. If 1 or 2 out of 4 rabbits tested showed reactions the result was recorded "irregular" neutralization, provided not less than 6 minimal doses of the reacting factors were present in each 1.25 cc. of the mixture. This conclusion was fully justified since the control titrations with this amount of reacting factors showed from 70 to 90 per cent of positive rabbits (Table I). If 3 out of 4 or all rabbits tested showed reactions there was recorded "no neutralization."

Table II represents the results of neutralization experiments with various amounts of reacting factors of different toxic filtrates mixed with constant amounts of certain immune sera.

As is seen from Table II and Diagrams 1 and 2, two types of neutra-

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bits may be amazingly small. In one instance, 0.0009 cc. of the filtrate was capable of eliciting a severe hemorrhagic necrosis at the prepared skin site. If one also considers that this amount was diluted in at least 100 cc. of rabbit's blood and that a part of it must have been lost in the tissues before reaching the prepared skin site, the amount actually capable of inducing the lesion is less than 0.000009 cc.

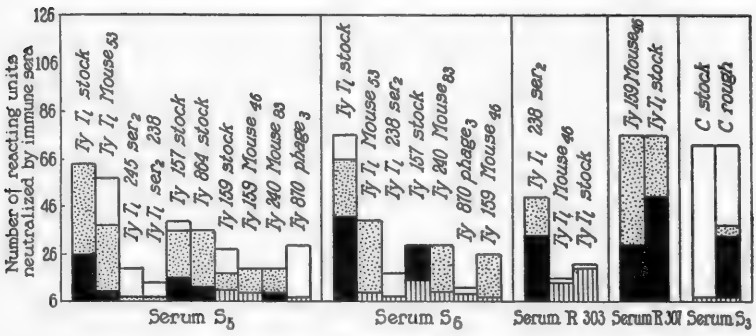

DIAGRAM 1

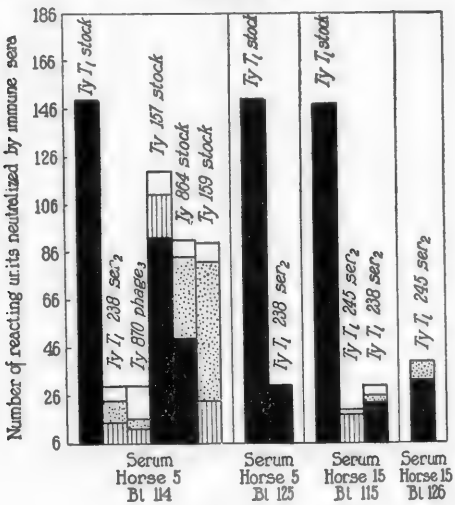

DIAGRAM 2

▥ = Not tested
▨ = Irregular neutralization
□ = No neutralization
■ = Complete neutralization

TABLE II

Serum Neutralizability of Various Bacterial Filtrates

No. of filtrate	Strain used for preparation of filtrate	No. of serum used for neutralization	Strain used for preparation of serum	Degree of neutralization of various amounts of reacting units by means of immune sera		
				Consistent neut.	Irregular neut.	No neut.
A_{26}	Ty T_L stock	S_5	Ty T_L stock	16,* 23, 24	30, 63	=
834	Ty T_L 245 ser_2	S_5	Ty T_L stock	—	6	11, 13, 19
823	Ty T_L 238 ser_2	S_5	Ty T_L stock	—	6	9, 12
839	Ty T_L $Mouse_{53}$	S_5	Ty T_L stock	9	10, 27, 38	58
852	Ty 157 stock	S_5	Ty T_L stock	13, 16	17, 20, 25, 33	40
846	Ty 864 stock	S_5	Ty T_L stock	12	14, 20, 26, 35	=
848	Ty 159 stock	S_5	Ty T_L stock	—	12	25, 28
971	Ty 159 $Mouse_{46}$	S_5	Ty T_L stock	—	9, 17, 20	=
911	Ty 870 $phage_3$	S_5	Ty 240 $Mouse_{60}$	—	—	8, 10, 15, 30
980	Ty 240 $Mouse_{53}$	S_5	Ty T_L stock	10	14, 20	=
A_{26}	Ty T_L stock	S_6	Ty 240 $Mouse_{60}$	8.5, 12.5, 18.5, 26.5, 30	55	75
823	Ty T_L 238 ser_2	S_6	Ty 240 $Mouse_{60}$	—	—	8, 12, 18
852	Ty 157 stock	S_6	Ty 240 $Mouse_{60}$	15, 30	=	=
971	Ty 159 $Mouse_{46}$	S_6	Ty 240 $Mouse_{60}$	—	8, 12, 18, 26	=
839	Ty T_L $Mouse_{53}$	S_6	Ty 240 $Mouse_{60}$	—	10, 20, 40	=
980	Ty 240 $Mouse_{53}$	S_6	Ty 240 $Mouse_{60}$	—	10, 20, 30	=
911	Ty 870 $phage_3$	S_6	Ty 240 $Mouse_{60}$	—	—	10
989	Ty T_L 245 ser_2	R-303	Ty T_L R-238 ser_2	12, 24, 36	50	=
971	Ty 159 $Mouse_{46}$	R-303	Ty T_L R-238 ser_2	—	—	15
A_{26}	Ty T_L stock	R-303	Ty T_L R-238 ser_2	—	—	20
A_{26}	Ty T_L stock	R-307	Ty 159 $Mouse_{40}$	25, 50	75	=
971	Ty 159 $Mouse_{46}$	R-307	Ty 159 $Mouse_{40}$	15, 25	35, 50, 75	=

A_{30}	Ty T_L stock	$Horse_4$ Bl_{100}	Polyvalent (1)	12, 24, 48, 85, 100, 140	150, 300	=
779	Ty T_L $Mouse_{43}$	$Horse_4$ Bl_{100}	Polyvalent (1)	14, 26, 53	75	100, 200
778	Ty T_L 238 ser_2	$Horse_4$ Bl_{100}	Polyvalent (1)	12	25	48, 94, 115, 150, 175, 235, 350
A_{26}	Ty T_L stock	$Horse_4$ Bl_{102}	Polyvalent (2)	120	=	=
823	Ty T_L 238 ser_2	$Horse_4$ Bl_{102}	Polyvalent (2)	10	15	=
A_{26}	Ty T_L stock	$Horse_5$ Bl_{114}	Polyvalent (3)	90	140	=
823	Ty T_L 238 ser_2	$Horse_5$ Bl_{114}	Polyvalent (3)	—	15, 20	30
852	Ty 157 stock	$Horse_5$ Bl_{114}	Polyvalent (3)	90	—	110, 120
846	Ty 864 stock	$Horse_5$ Bl_{114}	Polyvalent (3)	40	60, 80	90
848	Ty 159 stock	$Horse_5$ Bl_{114}	Polyvalent (3)	—	25, 40, 60, 75	90
911	Ty 870 $phage_3$	$Horse_5$ Bl_{114}	Polyvalent (3)	—	12	20, 30
A_{26}	Ty T_L stock	$Horse_5$ Bl_{125}	Polyvalent (12)	150	=	=
823	Ty T_L 238 ser_2	$Horse_5$ Bl_{125}	Polyvalent (12)	30	=	=
A_{26}	Ty T_L stock	$Horse_{15}$ Bl_{115}	Polyvalent (6)	90, 147	=	=
834	Ty T_L 245 ser_2	$Horse_{15}$ Bl_{115}	Polyvalent (6)	—	—	18
823	Ty T_L 238 ser_2	$Horse_{15}$ Bl_{115}	Polyvalent (6)	20	25	30
989	Ty T_L 245 ser_2	$Horse_{15}$ Bl_{115}	Polyvalent (6)	—	15	25, 35
911	Ty 870 $phage_3$	$Horse_{15}$ Bl_{115}	Polyvalent (6)	—	12	20, 30
989	Ty T_L 245 ser_2	$Horse_{15}$ Bl_{126}	Polyvalent (11)	15, 25	40	=
1042	C stock	S_2	C 42	—	—	8, 14, 24, 30, 40, 50, 60
985	C rough	S_2	C 42	14, 24, 30	35	40, 50, 60, 8

— = lower amounts not tested.

= = higher amounts not tested.

The horses were immunized with mixtures of vaccines and filtrates of various stock strains for a period of 8 to 12 months. Recently mixtures of vaccines and filtrates derived from various rough strains of *B. typhosus* were added to the material injected. The word "polyvalent" indicates injections of mixtures of stock strains, the number following indicates the number of injections of the rough strains.

* Each of these figures represents the number of units tested for neutralization in a group of 4 rabbits.

lizations were obtained, *i.e.*, consistent and irregular neutralizations. The irregular neutralizations which take place when the amount of reacting factors is increased beyond a certain maximum are most likely due to the fact that the mixtures contain a small amount of free reacting factors so that only the rabbits possessing a high degree of reactivity are able to respond with reactions. Quantitative comparisons of the degree of neutralizability of reacting factors derived from various filtrates can be made accurately by determining the largest amount of reacting factors *consistently* neutralized by a given serum. It does not appear safe to make quantitative estimations of the neutralizability of a filtrate by determining the largest amount of reacting factors *irregularly* neutralized by a serum since the exact mechanism of irregular neutralization is not known as yet. However, this type of neutralization offers a rough quantitative comparison of the degree of neutralizability of various filtrates by a given serum. If an analysis of this sort is made on the data obtained, the following becomes evident:

Various stock strains of *B. typhosus* show striking differences in the serum neutralizability of their reacting factors. Out of four strains chosen for this work two occupied extreme positions, one showing a high degree of neutralizability with strain homologous and strain heterologous sera (Ty T_L stock and Sera S_5 and S_6) and another strain (Ty 159 stock) showing no consistent but only irregular neutralizations with the same sera. A horse serum ($Horse_6$ $Bleeding_{114}$) which was prepared by injection of mixtures of various strains including Ty 159 stock also failed to give consistent neutralizations with Ty 159 stock reacting factors, although giving a high degree of irregular neutralization. The remaining two stock strains occupied intermediate positions in the degree of consistent neutralizations, one (Ty 864 stock) being of lower neutralizability than the other (Ty 157 stock). The strains preserved the same differences when tested with the polyvalent horse serum ($Horse_6$ $Bleeding_{114}$). The present tentative explanation of these findings is as follows:

The reacting factors of the strain showing the highest degree of neutralizability appear to be the simplest in their antigenic structure, as well as of high antigenicity. The reacting factors of the remaining stock strains appear to represent various degrees of increasing anti-

genic complexity, the Ty 159 stock strain being the most complex one. The additional complicating components, however, have to be considered of low antigenicity, as is seen from the fact that polyvalent sera, in the preparation of which there was included Ty 159 stock strain, and certain homologous monovalent rabbit sera fail to give a high degree of neutralization with these factors. Further proof of this contention is brought out by the following work with mouse strains:

The passage of *B. typhosus* stock strains through mice may or may not affect the neutralizability of their reacting factors. In one strain which was considered above as the simplest in the antigenic structure of its reacting factors (Ty T_L stock) there occurred a partial loss of neutralizability (Ty T_L $Mouse_{53}$ and Sera S_5 and S_6). The fact may be interpreted as an acquirement of additional components. The factors of another strain considered as the most complex ones (Ty 159 stock strain) showed no change in the neutralizability of the reacting factors after 46 passages through mice. However, there occurred an increase in the antigenicity of the factors of this strain. This fact is well illustrated by the experiments with R-309 serum homologous for Ty 159 $Mouse_{46}$ strain which shows a high degree of consistent neutralization with the reacting factors of the latter.

The passage of a strain through mice is not necessarily accompanied by an increase in the antigenicity of the factors of low neutralizability. The Ty 240 stock strain which showed factors of low neutralizibility was tested with the anti-Ty T_L stock serum (S_5) as well as with an anti-mouse-passed-Ty 240-strain serum (S_6) (previous unrecorded experiments). Both sera showed low neutralizations with the reacting factors of this mouse strain. (Table II, Diagrams 1 and 2.)

As is also seen from Table II and Diagrams 1 and 2 there occurs an almost complete loss of neutralizability of reacting factors of the rough variants by the anti-stock sera. The change is illustrated by numerous experiments (Ty T_L 238 ser_2 and Ty T_L 245 ser_2, Ty 870 $phage_3$ *vs.* Sera S_5, S_6, $Horses_{5/114}$ and $_{15/115}$). The loss of neutralizability is associated with acquisition of a new antigenic specificity. The "rough" reacting factors are consistently neutralized by homologous sera and by polyvalent sera, in the preparation of which there were included the rough variants (Serum S_3 and *coli* rough; Typhoid rough strains and Sera R-303 and $Horses_{5/125,\ 15/126,\ 15/115}$). Moreover, the two *B. typhosus* rough variants (Ty T_L 238 ser_2 and Ty T_L 245 ser_2), both derived from the same stock strain (Ty T_L), and a third *B. typhosus* rough

variant (Ty 870 phage$_3$) derived from another stock strain (Ty 870) varied in the degree of their neutralizability by anti-rough sera (Horses $_{5/114}$ and $_{15/115}$). The question whether the "rough" reacting factors are able to stimulate antibodies against normal reacting factors is left open.

In the work which follows an attempt was made to correlate the above findings with the morphological appearance of colonies, agglutinability and virulence of the various strains and also with the serum precipitability and lethal effect of the filtrates studied.

II. Morphological Appearance of Colonies and the Neutralizability of Reacting Factors

As is seen from Part I of this paper the stock strains of *B. typhosus* produced reacting factors of various degrees of neutralizability. The Ty T_L stock strain, producing factors of high neutralizability and antigenicity and apparently the simplest in antigenic structure, was morphologically intermediately rough and was capable of prompt transformation into complete roughness. Studies on other similar strains are under way in order to determine whether this correlation can be definitely established. The reacting factors of the remaining stock strains which differed in neutralizability and were considered more complex did not display any difference in the colony morphology, all appearing normal. Neither were these strains transformed into rough variants in spite of persistent attempts. It appears, therefore, that strains of normal colony appearance differ in the neutralizability of reacting factors which they produce.

The different rough variants produced by passage through immune serum and bacteriophage appeared of morphologically identical colony structure, whilst they differed in the neutralizability of their reacting factors.

The passage of stock strains through mice which may or may not result in a change of neutralizability was not accompanied by any changes in the morphology of the colonies.

III. Serum Agglutinability of Various Stock Strains and Variants of B. typhosus and the Neutralizability of Reacting Factors

The tests were performed in the usual manner, except that 0.21 per cent NaCl solution was used for making serum dilutions for the agglu-

tination of the rough variants. The growth of rough variants on solid media used for the tests was suspended in distilled water, washed by centrifugalization and resuspended in 0.21 per cent NaCl solution.

TABLE III

Serum Agglutination of Various Strains of B. typhosus

Strain	Serum	Degree of agglutination with various dilutions of sera										
		1–25	1–50	1–100	1–200	1–400	1–800	1–1600	1–3200	1–6400	1–12800	1–25600
Ty T_L stock	S_5	4+	4+	4+	4+	4+	4+	4+	2+	=	=	=
Ty 157 stock	S_5	4+	4+	4+	3+	3+	2+	1+	—	=	=	=
Ty 159 stock	S_5	3+	3+	3+	2+	2+	2+	1+	1+	=	=	=
Ty T_L stock	S_6	4+	4+	4+	4+	4+	4+	4+	3+	=	=	=
Ty 157 stock	S_6	4+	4+	4+	3+	3+	3+	1+	—	=	=	=
Ty T_L stock	$Horse_5$ Bl_{114}	4+	4+	4+	4+	4+	4+	3+	3+	3+	1+	—
Ty 157 stock	$Horse_5$ Bl_{114}	4+	4+	4+	4+	3+	2+	±	—	—	—	—
Ty 864 stock	$Horse_5$ Bl_{114}	3+	3+	3+	3+	3+	3+	3+	3+	3+	1+	—
Ty 159 stock	$Horse_5$ Bl_{114}	3+	3+	2+	1+	—	—	—	—	—	—	—
Ty T_L $Mouse_{94}$	S_6	4+	4+	4+	4+	4+	4+	4+	3+	=	=	=
Ty T_L $Mouse_{94}$	S_5	4+	4+	4+	4+	4+	4+	4+	3+	=	=	=
Ty T_L $Mouse_{26}$	$Horse_4$ Bl_{100}	4+	4+	4+	4+	4+	4+	4+	4+	3+	2+	1+
Ty T_L $Mouse_{56}$	$Horse_4$ Bl_{100}	4+	4+	4+	4+	4+	4+	4+	4+	3+	1+	1+
Ty T_L stock	$Horse_4$ Bl_{100}	4+	4+	4+	4+	4+	4+	4+	4+	3+	2+	1+
Ty 159 $Mouse_{34}$	S_5	4+	4+	4+	3+	±	—	—	—	=	=	=
Ty 159 $Mouse_{51}$	S_5	4+	4+	3+	±	—	—	—	—	=	=	=
Ty T_L 238 ser_2	S_5	2+	—	—	—	—	—	—	—	=	=	=
Ty T_L 245 ser_2	S_5	2+	—	—	—	—	—	—	—	=	=	=
Ty 870 $phage_3$	S_5	2+	—	—	—	—	—	—	—	=	=	=
Ty T_L 238 ser_2	R 303	4+	4+	4+	4+	3+	3+	2+	2+	—	=	=
Ty T_L 238 ser_2	$Horse_5$ Bl_{114}	4+	4+	4+	4+	4+	4+	4+	3+	1+	—	—
Ty T_L 245 ser_2	$Horse_{15}$ Bl_{115}	4+	4+	4+	3+	3+	3+	1+	±	—	—	—
Ty T_L 238 ser_2	$Horse_{15}$ Bl_{115}	4+	4+	4+	4+	4+	3+	3+	2+	1+	—	—
Ty T_L 245 ser_2	$Horse_{15}$ Bl_{126}	4+	4+	4+	4+	4+	4+	3+	2+	±	—	—

= not tested in higher dilutions.

The results of the agglutination tests with the various cultures, as recorded in Table III bring out the following:

There are two modes of comparison of agglutinability of various strains by immune sera: (a) the agglutination titer, namely, the highest dilution of the serum with which agglutination is obtained, and (b) the degree of agglutination in various dilutions of sera. The agglutina-

tion titer of the stock strains cannot be correlated with the degree of neutralizability of reacting factors derived from the same strains. For instance, the agglutination titer of Serum S_5 is the same for Ty T_L stock and Ty 159 stock strains, whilst there is consistent neutralization of 26 reacting units of the first and no consistent neutralization of the latter strain. In another example, the reacting factors of Ty 157 stock strain are neutralized by serum $Horse_5$ $Bleeding_{114}$ to a higher titer than those of strain Ty 864 stock, but the agglutination titer is higher for the second than for the first strain. On the other hand, it appears that the degree of agglutination obtained in various dilutions of sera may be an indication of differences in neutralizability of stock strains of *B. typhosus*. Thus, should one arrange the strains in order of their comparative neutralizability and in order of the intensity of agglutination in low dilutions of sera the same order is obtained for both. Thus: 1.—Ty T_L stock, 2.—Ty 157 stock, 3.—Ty 864 stock, and 4.—Ty 159 stock would fall in this order. The above described observation is to be differentiated from previously reported results showing that the agglutinating and neutralizing antibodies do not run parallel in immune sera (10).

The loss of agglutinability due to passage of strains through mice is not an indication of a change in the neutralizability of the reacting factors. For, on the one hand, there is the example of the Ty T_L mouse strains which, after passage through mice, produced reacting factors of lower neutralizability without change in agglutinability. On the other hand, in contrast to this strain, Ty 159 mouse underwent a considerable loss in agglutinability without concomitant loss of neutralizability of its reacting factors.

As is seen from the results of agglutination tests with the rough variants of *B. typhosus* and from neutralization experiments of Table II the anti-rough agglutinating and neutralizing antibodies do not run parallel. In fact, serum $Horse_5$ $Bleeding_{114}$ which was not able to neutralize consistently the reacting factors of strain Ty T_L 238 ser_2 has a higher agglutination titer than Serum R-203 which shows consistent neutralization of 43 reacting units of the same filtrate.

As noted before, various degrees of neutralizability exist among rough variants derived from the same stock strain. There appears to be parallelism between this and the agglutinability of the same vari-

ants by immune sera. ($Horse_{15}$ $Bleeding_{115}$ *vs.* Ty T_L 238 ser_2 and Ty T_L 245 ser_2.)

No antigen analysis (*i.e.*, somatic and flagellar antigens of Theobald Smith (22) etc.) of the various strains employed has been made as yet.*

IV. Serum Precipitability of Culture Filtrates of Various Stock Strains and Variants of B. typhosus and Neutralizability of Reacting Factors

The results of precipitation tests with various filtrates and immune sera are summarized in Table IV.

TABLE IV

Serum Precipitation of Various Bacterial Filtrates

No. of filtrate	Strain used for preparation of filtrate	No. of serum	Strain used for preparation of serum	Degree of precipitation with various dilutions of sera							
				1-4	1-8	1-16	1-32	1-64	1-128	1-256	1-512
A_{26}	Ty T_L stock	S_5	Ty T_L stock	4+	3+	±	—	—	—	—	—
852	Ty 157 stock	S_5	Ty T_L stock	2+	2+	±	—	—	—	—	—
848	Ty 159 stock	S_5	Ty T_L stock	4+	2+	±	—	—	—	—	—
839	Ty T_L $Mouse_{53}$	S_5	Ty T_L stock	4+	4+	1+	±	—	—	—	—
971	Ty 159 $Mouse_{46}$	S_5	Ty T_L stock	4+	4+	±	±	—	—	—	—
823	Ty T_L 238 ser_2	S_5	Ty T_L stock	—**	—	—	—	—	—	—	—
834	Ty T_L 245 ser_2	S_5	Ty T_L stock	—**	—	—	—	—	—	—	—
A_{26}	Ty T_L stock	R 303	Ty T_L 238 ser_2	2+	1+	—	—	—	—	—	—
823	Ty T_L 238 ser_2	R 303	Ty T_L 238 ser_2	4+	4+	1+	±	—	—	—	—
989	Ty T_L 245 ser_2	R 303	Ty T_L 238 ser_2	4+	4+	2+	1+	—	—	—	—

** No precipitation was obtained with the filtrate previously diluted 1 to 4.

A quantitative analysis of the results cannot be safely made since several dilutions of the antigens were not used in every instance. The following can, however, be concluded:

The "rough" reacting factors which are not neutralized by antistock sera are also devoid of precipitinogens for these sera (Ty T_L 238 ser_2 and Ty T_L 245 ser_2 strains and Serum S_5).

* The very extensive literature on inagglutinability of recently isolated *B. typhosus* strains and antigen changes occurring in the course of dissociation is reviewed by Baerthlein (16) and Hadley (17).

The appearance of neutralizing antibodies for the "rough" reacting factors is coincident with formation of anti-"rough" precipitins (Ty T_L 238 ser_2 and Ty T_L 245 ser_2 and Serum R-303).

Abundant precipitation between the filtrate and serum may occur in spite of low neutralization (Ty 159 stock and $Mouse_{46}$ and Serum S_6).

V. Virulence of B. typhosus Variants and Neutralizability of B. typhosus Reacting Factors

The various strains tested were transplanted into plain broth pH 7.4. 1 cc. of a broth culture previously diluted in sterile plain broth 1:4 was

TABLE V

The Virulence of Variants of B. typhosus

Total number of mice	Strain employed	Amount injected intraperitoneally	Number of deaths after various intervals of time				Survivals
			3 hours	24 hours	48 hours	90 hours	
25	Ty T_L stock	1 cc. of 1:5 dilution	2	18	1	0	4
25	Ty T_L $Mouse_{88}$	" "	0	14	3	0	8
25	Ty T_L 238 ser_2	" "	1	4	4	0	16
25	Ty T_L 245 ser_2	" "	0	0	1	7	18
25	Ty T_L stock	" "	0	9	1	7	8

injected intraperitoneally into each mouse. A large number of mice were tested in view of the considerable individual variations of the laboratory animals in response to the effect of the colon-typhoid group. The results are summarized in Table V. As is seen from Table V, the passage of the Ty T_L stock strain through mice did not increase its virulence. It is clear, therefore, that the change in the neutralizability of the reacting factors which occurred in the mouse strain is not related to its virulence.

There is an unquestionable lowering of virulence on the part of the rough variants of *B. typhosus*, as compared to the virulence of the stock strain from which they are derived. This was previously observed for the colon-typhoid group by Topley and Ayrton, Jordan, Orcutt, White, Goyle, Schütze and others (17). As is seen from this

paper, the lowering of virulence is coincident with a change in the neutralizability of the "rough" reacting factors by anti-stock sera.

TABLE VI

Lethal Effect of B. typhosus Culture Filtrates upon Mice

No. of mice tested	Amount of filtrate injected intraperitoneally	Strain used for preparation of filtrate	No. of filtrate	No. of deaths after various intervals of time				No. of survivals
				3 hours	24 hours	48 hours	52 hours	
	cc.							
25	1	Ty T_L stock	A_{26}	3	17	3	0	2
28	1	Ty T_L $Mouse_{53}$	834	9	16	2	0	1
28	1	Ty T_L 238 ser_2	823	8	16	0	0	4
25	1	Ty T_L 245 ser_2	834	4	8	0	3	10

TABLE VII

Lethal Effect of B. typhosus Culture Filtrates upon Rabbits

No. of filtrate	Strain used for preparation of filtrate	Intravenous dose per kilo of body weight	No. of rabbits tested	No. of deaths 1–5 hours after intravenous injection	No. of survivals
A_{26}	Ty T_L stock	1 cc. of 1:100 dil.	4	1	3
A_{26}	Ty T_L stock	1 cc. of 1:200 dil.	4	1	3
A_{26}	Ty T_L stock	1 cc. of 1:400 dil.	4	1	3
911	Ty 870 $phage_3$	1 cc. of 1:300 dil.	4	1	3
823	Ty T_L 238 ser_2	1 cc. of 1:400 dil.	4	1	3
834	Ty T_L 245 ser_2	1 cc. of 1:200 dil.	4	1	3
834	Ty T_L 245 ser_2	1 cc. of 1:800 dil.	4	1	3
989	Ty T_L 245 ser_2	1 cc. of 1:40 dil.	4	1	3
989	Ty T_L 245 ser_2	1 cc. of 1:20 dil.	4	1	3
840	Ty 159 $Mouse_{31}$	1 cc. of 1:800 dil.	4	1	3
840	Ty 159 $Mouse_{31}$	1 cc. of 1:400 dil.	4	1	3
852	Ty 157 stock	1 cc. of 1:300 dil.	4	1	3
852	Ty 157 stock	1 cc. of 1:500 dil.	4	1	3
852	Ty 157 stock	1 cc. of 1:600 dil.	4	1	3
852	Ty 157 stock	1 cc. of 1:110 dil.	5	1	4

However, there were also differences in the neutralizability of the reacting factors derived from the two "rough" variants by anti-rough sera. No differences could be recognized in the virulence of these rough variants.

VI. The Lethal Effect of B. typhosus Culture Filtrates and Neutralizability of Reacting Factors

In these experiments mice were injected with 1 cc. of each undiluted filtrate intraperitoneally. The rabbits* received each a single intravenous injection of diluted filtrate.

As is seen from Table VI, culture filtrates of variants of *B. typhosus* as well as the stock strain from which they were derived were decidedly toxic for mice. It may also appear that the filtrate of one of the rough variants was less than the other (Ty T_L 245 ser_2 and Ty T_L 238 ser_2 respectively). However, no conclusion has been drawn as yet in view of the extreme variations in the individual susceptibility of laboratory animals to the effect of bacterial filtrates.

As is seen from Table VII, even high dilutions of filtrates irrespective of the strains and the type of variants used for their preparation proved lethal to a small percentage of rabbits. It is evident from the data brought out here that no relationships can be established between the lethal effect of *B. typhosus* filtrates and the degrees of serum neutralizability of the reacting factors present in these filtrates. There is an apparent discrepancy between the results on the lethal effect of toxic substances derived from rough cultures of the colon-typhoid group obtained by the present author and previous authors. Thus, Goyle (18) found that the filtrate of the autolyzed culture of the normal strain of *B. enteriditis* is more toxic than that of the rough strain, the tests being made in 6 mice. Schütze (19) tested washed saline suspensions of bacteria heated to 60°C. and 100°C. in mice. One mouse was inoculated with each dilution. He concluded that the rough variant of S-Aertrycke-Glasgow possessed about a quarter of the toxicity of its smooth counterpart. Heating to 100° lowered the toxicity of the S-variant by about a quarter but the toxins of the rough variant did not appear to be so labile.

The discrepancy of results may be explained by the fact that the mode of preparation of filtrates was altogether different (6) from those employed by Goyle and Schütze and also by the fact that a considerably larger number of animals was used (because of individual variations

* The rabbits used in these experiments were previously injected intradermally for titration of the phenomenon-producing potency of the filtrates.

in susceptibility of animals). However, the results of White (20) support the present author's contention. He states as follows: "Saline suspensions of smooth heat-killed bacilli excel those of rough bacilli in toxicity. However, alcohol-treated rough *B. typhosus* and Salmonella bacilli exhibit for rats and rabbits a toxicity which hardly falls short of that of smooth. It seems probable that the potential toxicity of the rough derivative approximates to that of the smooth parent organism and that contrasts in actual toxicity are due to differences in the rate of liberation within the animal body or into fluid culture medium."

DISCUSSION

From previous studies it has been concluded that the phenomenon described is the result of a state of induced susceptibility to antigenically specific and neutralizable toxins from a great variety of microorganisms produced in tissues spontaneously resistant. Inasmuch as this phenomenon can be elicited in various organs and inasmuch as it is indicative of severe injury produced by specific bacterial toxic substances, it possibly expresses in "acute" form the underlying mechanism in the pathogenesis of spontaneous infectious diseases. In the light of this conception various problems of both practical and theoretical interest present themselves:

In previous studies the injury producing factors (*i.e.*, reacting factors) of the phenomenon under discussion were shown to be species specific. The work embodied in this paper demonstrates also strain and variant specificity.

By the "strain specificity" is meant the fact that various strains, chosen at random, of a given species of bacteria produce reacting factors which differ in their neutralizability. The differences are explainable on the basis of various degrees of antigenic complexity.

The "variant specificity" is demonstrated by a number of facts.

The passage through mice induces in certain strains a partial loss of neutralizability of reacting factors. This is probably due to variation towards smoothness with an increase in the antigenic complexity. As a matter of fact, the reacting factors of a strain considered as the simplest in their structure underwent a partial loss of neutralizability after passages through mice, whilst the neutralizability of reacting

factors of a strain found to be the most complex before the passage through mice was not influenced by the latter procedure. The other type of "variant" specificity is conditioned by the change of a strain into rough. The "rough" reacting factors acquire a new specificity.

The above described changes in the neutralizability of the reacting factors taken together cannot be consistently correlated with colony morphology, serum agglutinability and virulence of strains, nor with the serum precipitability and general toxicity of the culture filtrates. This subject has been taken up in detail in the text.

Inasmuch as the occurrence of bacterial variation *in vivo* is a recognized fact, the relationship established in this paper between the neutralizability of toxic substances and bacterial variation offers an opportunity to add new data to our immunological understanding of the course of typhoid fever and possibly other infectious diseases.

As seen previously, *B. typhosus* strains differ in their ability to undergo variation. If a given infection is produced by a "normal" strain incapable of changing into a "rough" one the toxic substances (*i.e* reacting factors) will be neutralized by the "normal" antibodies developing in the course of the disease. However, should the strain be capable of prompt change into "rough" the production of antibodies will promote the variation and there will appear in the body "rough" reacting factors non-neutralizable by the "normal" antibodies. Until now, the toxicity and change in specificity of "rough" bacterial filtrates have not been recognized. It was known, however, that once a strain becomes rough it loses its virulence and, therefore, the promotion of variation might be considered as a helpful process. The work embodied in the present paper demonstrates the injury producing effect of the "rough" bacterial substances both in general toxicity and in their effect upon prepared skin sites. It also demonstrates the change in their antigenic specificity. In the light of these facts it may be assumed that the course of a spontaneous infectious disease would also depend on the ability of a given strain to undergo variation, the disease being more protracted when such a change occurs, and lasting until the development of the anti-rough antibodies.

Inasmuch as the reacting factors of normal strains also vary in their neutralizability and the degree of their antigenicity, it might be ex-

pected that the course of the disease would also depend on these immunological properties of the invading organisms.*

It is probable that the above considerations will prove of importance in planning the serum therapy of infectious diseases. Assuming that they are so, it would seem necessary to prepare immune sera sufficiently potent to neutralize the "normal" reacting factors even of low neutralizability. For this purpose advantage could be taken of the above described increase in antigenicity obtained by means of mouse passage. The serum should also contain antibodies specific for "rough" reacting factors since the presence of such factors may be expected *a priori* in any disease running a protracted course and since the injection of anti-normal antibodies will promote their appearance, provided the invading organism is capable of the transformation. The author is attempting to prepare in this manner antityphoid and anti-*coli* immune sera for ultimate therapeutic use.

CONCLUSIONS AND SUMMARY

In this paper there is reported the effect of bacterial variation upon the reacting factors of the phenomenon of local skin reactivity to bacterial culture filtrates. By means of the method described in the text the following results were obtained:

Various stock strains of *B. typhosus* were found to produce reacting factors which differed in their neutralizability by anti-stock immune sera. The reacting factors of low neutralizability also possessed low antigenicity. The passage of strains through mice brought about in one instance a lowering of neutralizability of reacting factors and in another instance had no effect. It was possible to increase the antigenicity of the reacting factors by passage of the strain through mice.

* It is of interest to mention here the work by Durham (21), who many years ago assumed that the original typhoid infection is produced by various strains of allied bacilli. According to his view, if the different strains are similar, antibodies are produced to the several strains and no relapses occur. If a particular strain predominates in the primary attack, though effective antibodies to this strain may be produced and may bring the attack to an end, few or perhaps no antibodies will be produced to the less abundant strains. These strains may then be responsible for a relapse.

Transformation of "stock" strain into "rough" brought about the formation of reacting factors of new specificity. The "rough" reacting factors were neutralized by homologous sera and also differed in their neutralizability.

Reacting factors of various degrees of neutralizability were produced by stock strains which did not display any difference in colony appearance (with one exception). The "rough" reacting factors appeared in strains of typical "rough" colony morphology. However, "rough" strains of the same degree of roughness morphologically differed in the neutralizability of their reacting factors. The changes in neutralizability elicited by passage through mice had no influence on colony morphology.

There seemed to be a parallelism between the degree of agglutinability of stock strains by anti-stock sera and the degree of neutralizability of reacting factors of the same strain.

It was possible to obtain a partial loss of neutralizability by passage through mice with and without change in agglutinability. The agglutination titer of anti-rough serum was not an indication of its anti-rough neutralizing properties.

The degree of neutralizability of "rough" reacting factors paralleled the specific serum agglutinability of the strains producing them.

Abundant precipitation occurred in mixtures of toxic filtrates and immune serum in spite of low neutralization. The filtrates of rough cultures non-neutralizable by anti-stock sera failed to precipitate with the latter. The appearance of neutralizing anti-rough antibodies was coincident with the development of anti-rough precipitates.

The rough strains possessed a lower virulence than the stock strains from which they were derived. However, there was no difference in the virulence of "rough" strains, the reacting factors of which differed in their neutralizability. The filtrates containing "rough" reacting factors were seemingly as lethal to mice and rabbits as were filtrates of stock cultures of *B. typhosus*.

The possible relation of these findings to the immunological understanding of the course of infectious diseases and to serum therapy is discussed.

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